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AIMING AT MALARIA ELIMINATION IN ZANZIBAR

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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In memory of Ali Khamis Abbas; A wonderful colleague and a great friend

PREFACE

I started doing research when I was eleven years old. It was the summer of 1989, one year earlier; the Iraqi army had carried out a campaign against the Kurdish population. The army destroyed thousands of villages and cities and 182 000 civilian Kurds were sent to the concentration camps. My parents, long time activists for the Kurdish cause, together with my elder siblings started a survey to collect information on the range of the damages of the 1988's genocide campaign. I remember very well sitting with the questionnaires on numbers of households per village, or whether the villages had a school or a mosque. Those who answered were either refugees who had survived the genocide campaign or members of the resistance force, the Peshmerga. Tired faces who with lots of sorrow answered questions about their home villages that did not exist anymore.

A few years later, with the escalating war, I too, had to flee. I ended up in Sweden, which became my second homeland where I studied medicine. When the opportunity to be a part of this wonderful project on malaria in Zanzibar occurred, it felt just as the right thing to do. Malaria is a face of injustice, a terrible disease that hits those who have no means, a threat that can be prevented if we did really wanted to.

The survey I participated in 1989 did not stop the genocide. Our loved ones, friends and relatives who were sent to the concentration camps never came back. But the reports based on our survey were used by many human right activists in their efforts to make the genocide against the Kurds internationally recognized and hopefully it will help preventing further atrocities against people elsewhere. In the same way, I hope that knowledge I have acquired while doing work, will make a small contribution to the on-going global efforts to reduce malaria burden. After all, this is the reason why we should do research.

ABSTRACT

Following the increase of the international funding for implementation of the combined malaria control strategies in the past decade, a significant reduction of malaria attributed morbidity and mortality has been achieved. Yet, malaria is still a severe threat to global health. In 2013, more than 200 million malaria cases causing the death of over 600 000 people were reported.

Zanzibar was among the first to implement artemisinin-based combination therapy (ACT) for malaria treatment, strengthened vector control measures including long lasting insecticide treated nets (LLINs) and indoor residual spraying (IRS) as well as rapid diagnostic test (RDT) for malaria diagnosis at peripheral health care facilities.

We assessed the effectiveness of malaria control tools and interventions for achieving malaria elimination in Zanzibar by studying the temporal trends of different malariometric indices in two districts of Zanzibar (Micheweni and North A) with a population of approximately 100,000 people each, between 1999 and 2013. Moreover, we conducted a health facility based study in the same districts for evaluation of RDT for malaria diagnosis including its performance within the integrated management of childhood illness (IMCI) algorithm as well as its field applicability as a source of parasite DNA for DNA extraction for molecular surveillance.

The interventions, with high sustained community uptake, were associated with major decline in malaria transmission most pronounced from 2004 to 2007, after which there appears to be a steady state. The cross-sectional survey in 2013 revealed a 97.0% reduction of *Plasmodium falciparum* prevalence when compared to 2003. Health facility data showed 96.0 % reduction of parasitologically confirmed malaria infections. All cause mortality among children under five decreased by 70%. Moreover, the general perception of reduced malaria burden by the caretakers was not associated with reduced adherence to the vector control measures.

RDT sensitivity against PCR and blood smear microscopy was relatively low (76.5% and 78.6%, respectively). Adherence to the RDT results was excellent (99.9%) and RDT performed well in the IMCI algorithm with equally high adherence among children under five as compared with other age groups. Further, RDT showed to be a good and reliable source of parasite DNA, useful for malaria case detection, molecular surveillance and RDT quality control.

During the conduct of the studies in this thesis, malaria elimination was not achieved in Zanzibar. However, following implementation of effective and sustainable tools and interventions with high coverage and uptake, Zanzibar has reached a state of malaria pre-elimination. Additional tools and interventions are necessary for further reduction of malaria transmission towards malaria elimination.

LIST OF SCIENTIFIC PAPERS

I. Björkman A*, **Shakely D*,** Ali AS, Morris U, Bhattarai A, Mwinyi MI, Abbas AK, Xu W, Cook J, Al-Mafazy A-W, Omar R, Mcha J, Rand A, Elfving K, Bennett A, Petzold M, McElroy P, Drakeley C, Mårtensson A. Pre-elimination achieved but residual malaria transmission calls for new malaria control strategies in Zanzibar (Manuscript)

* Shared first authorship – contributed equally to the work

II. Beer N, Abdullah SA, **Shakely D**, Elfving K, Al-Mafazy A-W, Msellem MI, Petzold M, Björkman A and Källander K. High effective coverage of vector control interventions in children after achieving low malaria transmission in Zanzibar, Tanzania. Malaria Journal 2013 12:38.

III. **Shakely D**, Elfving K, Aydin-Schmidt B, Msellem MI, Morris U, Rahila Omar, Xu Weiping, Max Petzold, Bryan Greenhouse, Kimberly A. Baltzell, Abdullah S. Ali, Anders Björkman, Andreas Mårtensson. The Usefulness of Rapid Diagnostic Tests in the New Context of Low Malaria Transmission in Zanzibar. PLoS ONE, 2013 Sept 4; 8(9)

IV. Morris U, Aydin-Schmidt B, **Shakely D**, Mårtensson A, Jörnhagen L, Ali AS, Msellem MI, Petzold M, Gil JP, Fereira PE, Björkman A. Rapid diagnostic tests for molecular surveillance of Plasmodium falciparum malaria -assessment of DNA extraction methods and field applicability. Malaria Journal 2013 12:106

Articles not included in this thesis:

Fröberg G, Jörnhagen L, Morris U, **Shakely D**, Msellem MI, Gil JP, Björkman A, Mårtensson A. Decreased prevalence of Plasmodium falciparum resistance markers to amodiaquine despite its wide scale use as ACT partner drug in Zanzibar. Malar J. 2012 Sep 11;11:321.

Baltzell KA, **Shakely D**, Hsiang M, Kemere J, Ali AS, Björkman A, Mårtensson A, Omar R, Elfving K, Msellem M, Aydin-Schmidt B, Rosenthal PJ, Greenhouse B. Prevalence of PCR detectable malaria infection among febrile patients with a negative Plasmodium falciparum specific rapid diagnostic test in Zanzibar. Am J Trop Med Hyg. 2013 Feb; 88(2):289-91.

Baltzell K, Elfving K, **Shakely D**, Ali AS, Msellem M, Gulati S, Mårtensson A. Febrile illness management in children under five years of age: a qualitative pilot study on primary health care workers' practices in Zanzibar.
Malar J. 2013 Jan 28;12:37

Aydin-Schmidt B, Xu W, González IJ, Polley SD, Bell D, **Shakely D**, Msellem MI, Björkman A, Mårtensson A. Loop mediated isothermal amplification (LAMP) accurately detects malaria DNA from filter paper blood samples of low density parasitaemias.

PLoS One. 2014 Aug 8;9(8).

CONTENTS

LIST OF ABBREVIATIONS

ACT	Artemisinin Combination Therapy
An	Anopheles
BS	Blood smear
CI	Confidence interval
Ct	Cycle threshold
Cyt b	Cythochrome b
DDT	Dichlordiphenyltrichloroethane
dNTP	Deoxynucleotide triphosphate
EIR	Entomological inoculation rate
ELISA	Enzyme-linked immunosorbent assay
FIND	Foundation of innovative new diagnostics
GAMP	Global malaria action plan
GMEP	Global Malaria Eradication Program
HBR	Human biting rate
IMCI	Integrated management of childhood illnesses
IPTp	Intermittent preventive treatment in pregnancy
IRS	Indoor residual spraying
ITN	Insecticide treated bed net
LAMP	Loop Mediated Isothermal Amplification
LLIN	Long lasting insecticide treated net

MA	Malaria Admission
MAD	Malaria Attributed Death
P/μL	Parasite/ microliter
PCR	Polymerase Chain Reaction
PfAMA-1	*Plasmodium falciparum* apical membrane antigen-1
Pfcrt	*Plasmodium falciparum* Chloroquine resistance transporter gene
PfGLURP	*Plasmodium falciparum* glutamate rich protein
PfHRP2	*Plasmodium falciparum histidine reach protein 2*
Pfmdr1	*Plasmodium falciparum* multidrug resistance 1 gene
PfMSP-1	*Plasmodium falciparum* merozoite surface protein
pLDH	*Plasmodium* Lactate Dehydrogenizes
qPCR	Real time PCR
RBC	Red blood cell
RBM	Roll Back Malaria
RDT	Rapid Diagnostic Test
RFLP	Restriction Fragment Length Polymorphism
Rp	Pearson Correlation Coefficient
rRNA	Ribosomal RNA
SCR	Seroconversion Rate
SNP	Single Nucleotide Polymorphisms
SP	Sulfadoxine-Pyrimethamine

WBC	White Blood Cell
WHO	World Health Organisation
ZAMEP	Zanzibar Malaria Elimination Program
ZMCP	Zanzibar Malaria Control Program
μL	Microliter

1 BACKGROUND

1.1.1 General introduction to malaria

Malaria is one of the oldest described diseases. The first written evidence of malaria is found in the Chinese medical classic Nei Chin (the Canon of Medicine) from 2,700 BC (**1**).

The malaria parasite is believed to have had the largest selective impact in recent history of the human genome. Many diseases associated with erythrocyte defects such as sickle-cell anaemia, thalassemia and glucose-6-phosphatase deficiency have been driven by the evolutionary power of malaria (**2**).

Due to its complicated life cycle and the its ability to be transmitted by a vector with high surviving skills, human beings still have not been able to defeat this disease that has killed famos men and women, emperors and warriors, writers and artists and most significantly millions of ordinary people around the world.

During last few years and following the increased funding for malaria control efforts, significant reduction of the global malaria burden has been achieved. Since year 2000, malaria prevalence and malaria attributed mortality has been reduced by nearly 50%. Reports from several countries in Africa have shown a significant decline of malaria incidence following deployment of the new global strategy (**3-13**). Yet, in 2013, an estimated number of 200 (124-283) million malaria cases were reported causing the death of 600 000 to one and half million people. The majority (90%) of all malaria deaths occurred in Africa, mostly among children under five who accounted for ca. 80% of all malaria attributed deaths (**14-15**).

Malaria is generally seen as “a disease of the poor” (**16**). The close association between malaria and poverty can be easily illustrated by a look at the global maps of poverty and malaria distributions. Unsurprisingly these two are to a large degree consistent with each other. People living in poverty spend less on malaria preventive measures such as bed-nets and afford less to visit health facilities for their malaria management and hence are at a higher risk for malaria transmission than less poor people. Importantly not only poverty can lead to an increased burden of malaria but malaria too, can cause poverty and deteriorate it. A lower malaria burden leads to positive economical development and can reduce poverty. As malaria affects some of the poorest people in the world, reducing malaria burden means a break in the cycle of poverty (**16-19**).

1.1.2 History

Malaria (from the Italian *mal'aria,* "bad air") received its widely accepted name by Romans who associated marshes around city of Rome with fever. Being good constructors, the Romans conducted a drainage program of the swamps around Rome as the first antimalarial intervention. However, malaria and its symptoms and causes have been described much earlier. Chinese, Indian, Mesopotamian and Ancient Greeks have all contributed to further understanding of malaria. Hippocrates described and classified malaria symptoms and the Sanskrit script by Sushruta suggested that malaria is caused by mosquito biting (**20**).

Important milestones in history of malaria were made in the late 19th century when Alphonse Laveran, a French army surgeon stationed in Algeria described the malaria parasite in human blood in 1880. Another army surgeon, British Ronald Ross discovered oocytes in the wall of the stomach of the Anopheles mosquito in India in 1897 and the following year, he described the complete life cycle of bird malaria in naturally infected sparrows in Kolkata, India. The same year Italian Giovanni Grassi and his colleagues described the cycle of human malaria parasites in *Anopheles* mosquitoes and showed that the sexual phase of *Plasmodium* takes place only in the *Anopheles* mosquito (21).

1.2 THE MALARIA PARASITE

The organism causing malaria is a unicellular parasite, a protozoon of *Plasmodium* genus. There are five species of *Plasmodium* that can infect human beings: *P. falciparum, P. vivax, P. ovale*, *P. malariae* and *P. knowlesi*

1.2.1 P. falciparum

P. falciparum is the dominant *plasmodium* species in malaria endemic areas of Africa. It is also found in other tropical and subtropical regions outside Africa. *P. falciparum* is regarded as the most severe *plasmodium* causing the highest rate of malaria morbidity as well as over 90% of all malaria mortality (**22**). This thesis is mainly focusing on *P. falciparum* as it is the dominant malaria species in Zanzibar.

1.2.2 P. vivax

P. vivax is the most geographically spread species of five human malaria species. It is the dominant *plasmodium* species in The Americas, Southeast Asia, Middle East and the Pacific region (**23**). *P. vivax* needs Duffy blood group antigen on the surface of the red blood cells (RBC) to invade the cell. The common prevalence of Duffy negative blood group among the African population is believed to be the natural selection for rare prevalence of this *Plasmodium* species in major parts of Sub-Saharan Africa especially in West Africa (**236**). *P. vivax* has the ability to be metabolically inactive and transform into a so-called hypnozoite form and relapse several months after its entry into human blood.

1.2.3 P. ovale and P. malaria

These two species are less common compared to the previous presented species. *P. ovale* is mostly found in Africa (West Africa) and the Pacific region (**24**) *P. ovale* is able to develop into hypnozoite and relapse months and even years after the initial infection.

P. malariae's distribution is similar to *P. ovale* but it can also be found in most other malaria endemic areas such as in Zanzibar.

1.2.4 P. knowlesi

This is the least common species of malaria mostly found in certain regions of South-East Asia. *P. knowlesi* is naturally hosted by macaques monkeys. Zoonotic transmission was until recently limited but today *P. knowlesi* is the main cause of malaria in Malaysia especially on the island of Borneo (**25**) Up to now, there is no evidence of development human-to-human transmission of *P. knowlesi* (**26**).

1.3 MALARIA VECTOR

The only mosquito genus able to transmit human malaria infection is *Anopheles* that contains more than 400 species of which 70 are malaria vectors (**237**). The life cycle of *Anopheles* is divided into four stages: eggs which are laid on the surface of water and hatch after ca. 48 hours into larvae which develops later into pupae and finally to adult mosquitoes. The total life span is up to four weeks and the first three stages are dependent on temperature (**27**). There are several different *Anopheles* species in various geographical settings with different capacities of malaria transmission. *Anopheles* mosquito can be found worldwide except in Antarctica and the Pacific islands east of the Vanuatu (the Buxton line) (**28**). Historically, malaria has been a global disease. Even in northern Europe, malaria transmission was relatively common until the first decades of the 20th century. In Sweden, malaria was eliminated first in 1930s (**74**). However, the most suitable temperature for parasite development inside *Anopheles* is between 25°-30° C and it normally ceases when the temperature is below 16° C and above 35° C (*P. falciparum* can not complete its growth cycle inside an *Anopheles* mosquito in temperatures < 20° C). The suitable temperature for development of *Anopheles* larvae is between 20°-30° C and the vector itself cannot survive a temperature > 42° C. Hence, malaria transmission mostly occurs in tropical and subtropical regions in the world on an altitude below 2000 meter (**29, 237**).

The feeding behaviour of the *Anopheles* mosquito is the crucial factor for its transmission capability. *Anopheles* mosquito usually takes its blood meal between dusk and down. However, there is a large variation in feeding behaviour among *Anopheles* species. There are early and late feeders. Some are indoor biters (endophagic) and some are outdoor biters (exophagic), some prefer bovine blood meals (zoophilic) and some prefer human blood meals (antropophilic). The dominant *Anopheles* species in Africa is *Anopheles Gambiae* complex, which is strongly antropophilic.

1.4 THE MALARIA LIFE CYCLE

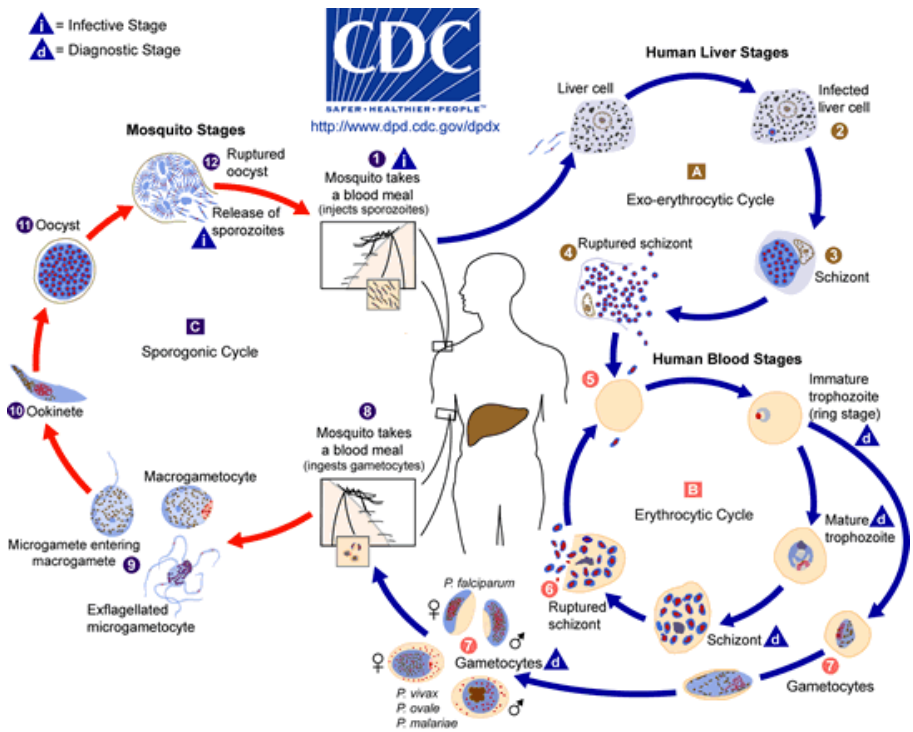
Figure 1 illustrates the life cycle of malaria parasite. The complexity of malaria transmission is partly due to its complicated life cycle involving three different actors: the parasite (*Plasmodium*), the vector (*Anopheles* mosquito) and the host (human being).

The life cycle of the malaria parasite begins when the female infected *Anopheles* mosquito bites the host to take a blood meal. The mosquito injects its saliva containing both anticoagulants and the asexual sporozoites into the blood vessel of the host. Via the blood stream, the sporozoites reach the liver and enter into the liver cells (hepatocytes) within 30 to 60 minutes. The rapid asexual replication of the sporozoites occurs inside the hepatocytes, turning them into mature liver schizonts containing up to 40 000 mature merozoites. This part of malaria infection which takes one to two weeks to be completed is asymptomatic and is known as the pre-erythrocytic phase. Both *P. vivax* and *P. ovale* can upon the invasion of the hepatocytes, enter into a low metabolic phase (hypnozoite) and remain inactive for weeks or even months before re-activation (relapse).

When the schizonts rupture, the merozoites that manage to escape the Kupffer cells (Macrophages inside the liver) are released into the blood stream and assisted by merozoite surface protein, they attach themselves to the erythrocytes, invade them and start the second

phase of malaria infection known as the erythrocytic phase. This is a rapid process and takes no longer than a minute. After the entry of the merozoites into the erythrocytes, asexual multiplications take place and erythrocytic schizonts consisted of 8-24 merozoites are built. Upon maturation, these schizonts rupture and merozoites and toxins made in the process are released into the blood stream. The released merozoites attack new erythrocytes and this chain reaction is the origin of the periodic fever attacks and shivering that is seen in some malaria patients. A fraction of the merozoites do not develop into erythrocytic schizonts but turn into sexual forms called gametocytes. Erythrocytes that contain gametocytes do not rupture and when another *Anopheles* mosquito takes a blood meal from an infected host, the gametocytes are transmitted to the vector. The fertilization takes place inside the gut of the mosquito. Thereafter, new oocytes containing sporozoites are created. Mature oocytes rupture and the sporozoites migrate to salivary glands of the mosquito. The cycles in completed when these sporozoites are injected into a human host when the next blood meal is taken (**30**).

Figure 1: malaria life cycle. Source: CDC http://www.cdc.gov/dpdx/malaria/



1.5 EPIDEMIOLOGY

In the mid 19th century, malaria transmission occurred almost worldwide. Today, despite progresses made in its control, malaria transmission affects around a hundred countries and territories around the world and puts more than three billion people at its risk (**15, 31**).

Malaria transmission can occur in any area where *Anopheles* mosquito can survive and multiply and human reservoir of malaria parasites can exist. The transmission can also happen without involvement of a vector. It can occur from a malaria-infected mother to her foetus during pregnancy (i.e. vertical transmission) or through blood transfusion.

1.5.1 Malaria endemicity

Classification of malaria endemicity i.e. quantifying the presence of malaria or its severity in a population or area; was historically done by measurement of splenomegaly (enlargement of the spleen) among children aged 2-9 years old in community based surveys. This was later revised to classification in relation to parasite rate among the population using blood smear (BS) microscopy (**238**).

According to this system malaria endemicity is divided into four categories based on splenomegaly or parasitaemia. These four categories are:

a) Holo-endemic (>75%): transmission occurs the whole year
b) Hyper-endemic (51-75%): periods of no transmission during the dry season
c) Meso-endemic (11-50%): regular seasonal transmission
d) Hypo-endemic (≤ 10%): intermittent, irregular malaria transmission

The entomological inoculation rate (EIR) i.e. number of infected *Anopheles* mosquito bites per person per unit of time is another method for classification of high and low malaria transmission settings. High malaria transmission is a setting where EIR>10 per year and low malaria transmission is a setting where EIR <1 per year (**239**).

Malaria transmission can also be classified into stable and unstable malaria. In a stable malaria setting, malaria transmission is high with no significant changes over years despite seasonal fluctuations. Stable malaria transmission settings can be found in holo- and hyper-endemic areas in Sub-Saharan Africa where *P. falciparum* is the dominant parasite species. Due to the high exposure to the infection, the population develops a high level of immunity.

In unstable malaria settings that are mostly found in hypo- and meso-endemic areas, malaria transmission changes from year to year. Due to fluctuation of malaria incidence, the level of immunity among the population is low. *P. vivax* is the most common malaria species in unstable malaria transmission settings. However outbreaks of *P. falciparum* can also occur in these settings (**237, 32**).

Figure 2: Percentage of people living under 2 US$ per day (1995-2013)

Source: World malaria report, 2014 (15)

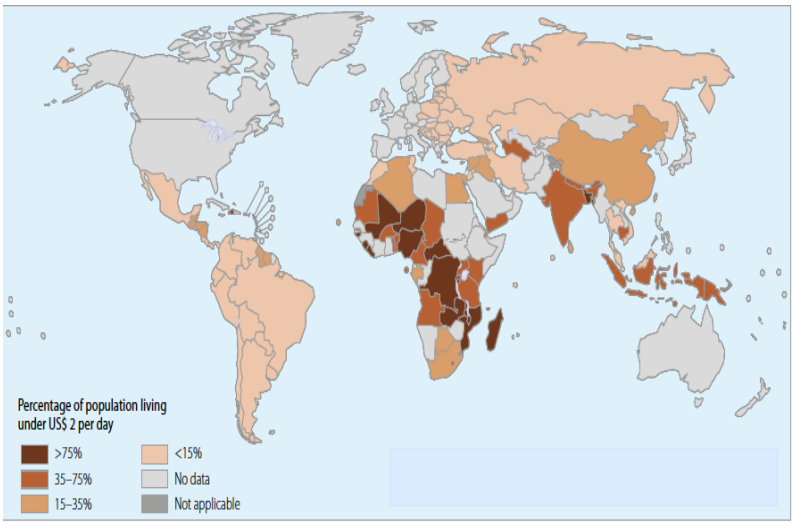


Figure 3:Malaria risk areas of the world from mid-19th century to the present.

Source: WHO, Global action plan for a malaria free world (18)

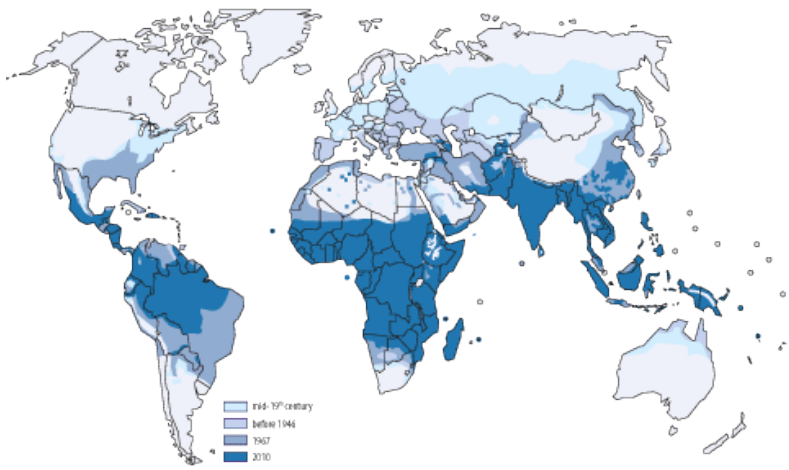


Figure 4**:** Country malaria classification

Classification of countries by stage of elimination, as of December 2013 Source: http://worldmalariareport.org/

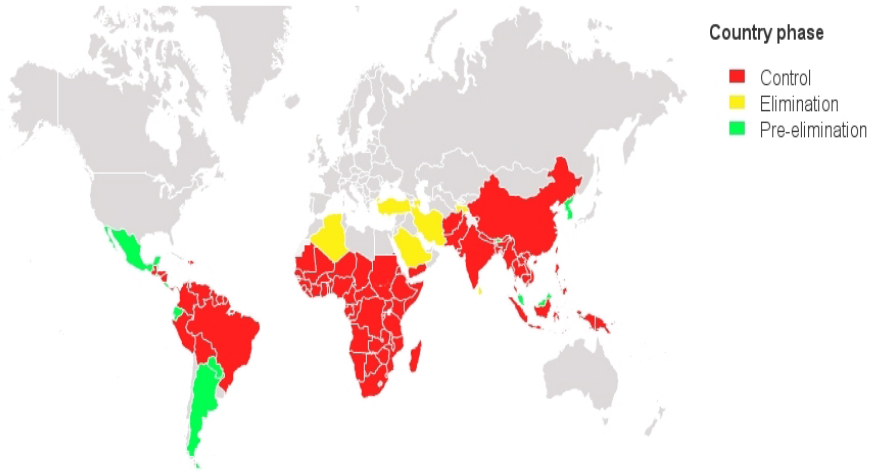
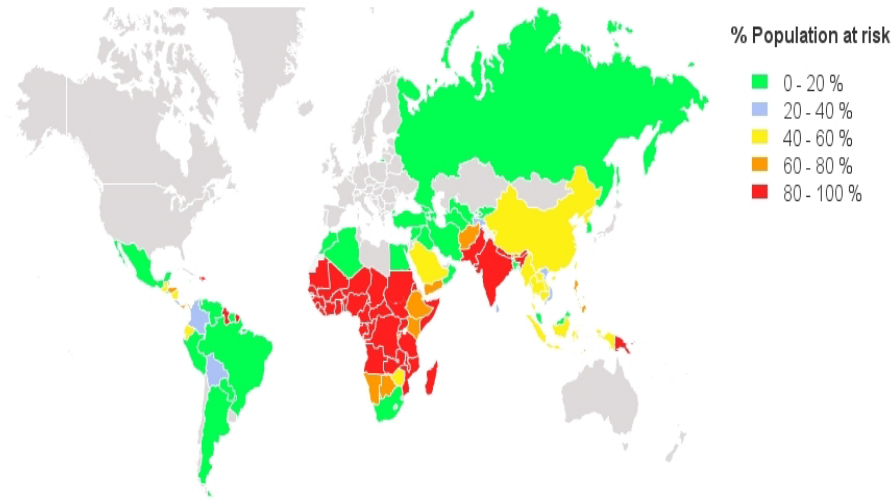


Figure 5: Percentage of Population at risk of malaria. Source: http://worldmalariareport.org/



1.6 CLINICAL FEATURES OF MALARIA

The cardinal symptom of malaria is fever. Hence, a patient living or coming from a malaria endemic area with a fever or a history of fever in the past 24-48 hours must always be investigated for malaria. Clinical manifestation of malaria is divided into complicated (severe) and uncomplicated malaria. All malaria species cause uncomplicated malaria but severe malaria is mainly due to *P. falciparum* infection. The uncomplicated malaria can develop into severe malaria.

The first clinical manifestation of malaria (*P. falciparum)* occurs earliest seven days after the initial infection. Malaria is a febrile disease with several unspecific symptoms such as headache, abdominal, chest, joint and muscle pain, nauseas and vomiting as well as general malaise. In some patients, the so called malarial paroxysm (quick attack or intensification of a malaria symptom, including an abrupt temperature elevation accompanied by heavy sweating which usually happens in intervals) occurs.

Uncomplicated malaria is defined as a patient with parasitaemia with one or several of following symptoms:

- Fever or history of fever
- Headache
- Cough
- Diarrhoea
- Muscle pain
- Vomiting

Complicated (Severe) malaria is defined as a patient with parasitaemia with one or several of the following symptoms:

- Impaired consciousness
- Multiple convulsions: more than two episodes in 24 hours
- Deep breathing and respiratory distress (acidotic breathing)
- Acute pulmonary oedema and acute respiratory distress syndrome
- Circulatory collapse or shock, Hypo-systolia i.e. systolic blood pressure < 80mm Hg in adults and < 50mm Hg in children
- Renal impairment (serum creatinine > 265 μmol/L)
- Abnormal bleeding
- Severe normocytic anaemia (Hemoglobin <50 g/L)
- Hyper-parasitaemia (2.5%- 20%)
- Hypo-glycaemia (< 2.2mmol/l or < 40mg/dL)
- Metabolic acidosis (plasma bicarbonate < 15 mmol/L)
- Hyper-lactataemia (lactate > 5 mmol/L)

(**33, 237**)

Severe malaria is mostly attributed to *P. falciparum* malaria infection. One reason behind this is the capability of the *P. falciparum* parasite to escape the immune cells by so called rosetting and sequestration. During the maturing stages of *P. falciparum*, cyto-adherent

proteins on the surface of the infected RBCs are expressed making them "sticky". The sticky surfaces of infected RBCs bind to uninfected RBCs and form the so-called rosettes (**34**). The cyto-adherent proteins also facilitate the sequestration process in which the infected RBCs bind to the endothelium of the deep vessels preventing them from incapacitation by the monocytes and macrophages in the spleen (**35**). Erythrocyte rosetting is crucial for parasite sequestration and development of cerebral malaria (**36**). This condition is characterized by rapid progression from headache to convulsions and coma and causes the death of hundreds of thousands of children in Africa annually (**37**).

The exact pathology of cerebral malaria remains unclear but the widely accepted hypothesis for the mechanism of the disease is the series of complicated inflammatory reactions including rosetting and sequestration of the infected RBCs resulting in dysfunction of the blood brain barrier. The rosettes clog the vessels in the brain and the blood circulation in the brain is deteriorated causing cerebral damages. Further, restriction of the venous blood flow caused by the process described above, exacerbates brain oedema resulting in intracranial hypertension (**37-39**). Others explain the pathophysiology of cerebral malaria due to the toxic effects of the overproduction of cytokines and other soluble mediators such as tumour necrosis factor (TNF-α) and interleukin (IL-1) or nitric oxide (NO) on the central nervous system causing coma (**40**). Cerebral malaria is a potentially fatal condition among both adults and children. In addition to cerebral malaria, severe anaemia and hypoglycaemia are also associated with malaria attributed mortality in children with severe malaria (**240-242**). In both adults and children, metabolic acidosis caused by obstruction of blood flow has shown to be the best independent predictor of fatal malaria. Metabolic acidosis is in up to 30% associated with a deadly outcome (**41,42**)**.** Neurological sequelae among children who survive cerebral malaria are manifested as memory impairment, ataxia, speech disorder and blindness (**43**).

1.6.1 Malaria and pregnancy

In high and moderate endemic areas where level of immunity among the population is high, the main impact of malaria on the pregnant women is anaemia, which can increase the risk for miscarriage, and perinatal death as anaemia caused by malaria infection contributes to maternal death as well as low birth weight (LBW=Birth weight <2500 g). LBW is the main risk factor for infant mortality. Pregnant women with little or no malaria immunity have a three-fold higher risk of developing severe malaria than non-pregnant women in the same area. (**243**). Regardless of the level of immunity, malaria infection increases the risk for both maternal and infant mortality. LBW occurs in both high and low endemic areas and among pregnant women with both high or low parasite density.

Malaria in pregnancy is characterized by an accumulation of infected RBCs in the placenta which is facilitated by the interaction between infected RBCs and the placental chondroitin sulphate A that fills the space between the villi containing the vessels of the mother and the embryo. This interaction causes disturbance in the transport of oxygen and nutrition to the foetus (**44-45**). Further, malaria in pregnancy is also associated with increased susceptibility to other infections than malaria with negative impact on both mother and the foetus (**46**).

Considering the above, prevention of malaria infection in pregnant women is critical both for saving lives of the mother and the child and to prevent the vertical transmission of malaria from the mother to the foetus. Use of intermittent preventive treatment in pregnancy (IPTp) at least twice during the pregnancy has resulted in a declined number of malaria infection cases, reduced proportion of low birth weights as well as lower death rates among both women and children (**47**). Today, the World Health Organisation (WHO) recommends treatment with IPTp with sulfadoxine-pyrimethamine (SP) at least twice during the pregnancy for all pregnant women who live in stable malaria transmission areas of sub-Saharan Africa (**244**).

1.7 MALARIA DIAGNOSIS

1.7.1 Clinical algorithm for malaria diagnosis

The initial symptoms of malaria are not specific. In areas where access to laboratory tools or skills is limited, symptom overlap between malaria and other febrile diseases, mainly acute respiratory infections especially in children, is a challenge for clinicians (**48-50**).

A study in Malawi showed that 95% of the children with the the clinical criteria for pneumonia also met the clinical criteria for malaria (**51**). Presumptive malarial treatment for all febrile patients in high malaria endemic regions has been practiced for a long time and conditioning antimalarial treatment only upon confirmation of malaria diagnosis has been and is a subject of debate (**52-55**).

As accurate laboratory methods for diagnosing the aetiology of febrile diseases are not available, overuse of both antimalarial and antibacterial drugs are common. Studies have shown that a decline of malaria transmission is followed by an increase of treatment with antibiotics (**56-57**). Improvement of malaria diagnosis becomes even more important when some recent studies on fever aetiology among febrile children suggest viruses and not bacteria being the main cause of fever among these patients (**58, Elfving et al, submitted**).

1.7.1.1 Integrated Management of Childhood illness

Integrated Management of Childhood illness (IMCI) is an algorithm used for management of illness in children aged 2–59 months in low and middle-income countries. IMCI's focus is not disease-specific. It is a guideline for health personnel at the first level health care facilities to classify conditions of an ill child through clinical assessment, classification, treatment, advice and follow up. The aim of IMCI is to decrease mortality and morbidity of children by improvement of the case management capacity of health system as well as the family and the community practices (**59**).

IMCI guides the health workers to assess the sick child in order to identify the danger sign/s, then classify the ill children into three categories:

- Ill children who must be urgently referred to a second level health care facility
- Ill children who can be managed by specific medical treatment and advice and
- Ill children who require only simple advice on home management

The caretaker receives practical advices regarding treatment. Depending on the child's condition, follow up instructions to the caretaker are provided (**60**). IMCI guidelines are

adapted for different regions and countries in order to cover the most common diseases in the specific regions and to be consistent with the national treatment guideline in order to facilitate their implementation with regards to the health system and the society in general (**61**).

Evaluation studies on IMCI effectiveness suggest that the IMCI guidelines increase quality of care, reduce overall costs as compared with vertical child health programs and in some setting, reduce childhood mortality (**62, 63**). Moreover, IMCI guidelines have shown to improve the use of antibiotics at the first level health care facilities (**64**) and the management of pneumonia, gastroenteritis, measles and malnutrition. However, when no BS microscopy was used for malaria diagnosis, a significant overtreatment with antimalarial medicines was observed (**65**).

1.7.2 Parasitological confirmed malaria diagnosis

Rapid diagnosis and treatment of a potential fatal disease as malaria is critical for prevention of death and other severe consequences due to the disease. Until recently, parasitological confirmation of malaria diagnosis in high endemic areas was not considered as cost effective and presumptive treatment of all fever cases was recommended **(237).** Following a significant increase of funds for malaria control during the 2000s and introduction of Rapid diagnostic test (RDT) for malaria diagnosis, WHO is now recommending parasitological confirmation of malaria diagnosis before treatment with antimalarial drugs **(66).**

1.7.2.1 Malaria microscopy

Light microscopy of stained BS is considered to be the gold standard in malaria diagnosis as this method has several advantages: it is relatively cheap, it differentiates between different malaria species, it can be used for quantification of parasite densities and it can identify different parasite stages (**67, 68**).

Parasite quantification is done by counting number of parasites observed in the thick smear against a standard number of white blood cells (WBC). In order to determinate the parasite density, the following formula is commonly used:

(Parasites counted/ number of WBC counted) X 8000= Parasites/µl

An alternative quantification method is, determination of parasite density as a percentage of infected RBCs on the thin film **(67, 69,70).**

The sensitivity of malaria microscopy under ideal conditions is very high with a detection limit down to 5-10 parasite/µl. But it is highly dependent on the microscopist and the quality of BS. In real life, at peripheral health care levels in low-income countries, the detection limit is about 100 parasite/µL (**71**). Since malaria microscopy is a time consuming and work intensive procedure, it can lead to fatigue and reduced diagnostic accuracy of the microscopist (**72**).

1.7.2.2 RDT

In 1990s, RDT was introduced as an alternative method for malaria diagnosis in febrile patients in settings where access to malaria microscopy was not optimal. Following this, the testing rate increased annually. In 2013, for the first time in Africa, the number of conducted

malaria diagnostic tests was higher than the number of prescribed artemisinin-based combination therapy (ACT) doses. As the number of malaria diagnosis by BS microscopy was not changed, the increased number of tested patients was attributed to use of RDTs for malaria diagnosis (**15**).

The detection limit of RDTs is almost equal to those of good field microscopy i.e. (~100 parasites/ μL) (**73**). However, there is a major variety in the sensitivity of different RDT products (**245**). The recommended level of sensitivity is ≥95% for detection of ≥100 p/μL and a minimum 90% for all malaria species compared with BS microscopy (**71, 75**). Moreover, several studies have shown a vast variety in the sensitivity of RDTs in the field have been shown in different studies (**56, 76-78**).

Basic principal of the malaria RDT is the detection of parasite antigens. Parasite antigens detected by RDTs are: Histidine-Rich Protein-2 (HRP-2), *Plasmodium* Lactate Dehydrogenizes (pLDH) and Aldolase-pan malaria antigen.

HRP-2: HRP-2 is a histidine- and alanine-rich protein localized in the *P. falciparum* cytoplasm as well as in the infected erythrocyte's membrane and is therefore the target for the RDT. It is a water-soluble and heat-stable protein that is synthesized only by *P. falciparum* parasites (258). HRP-2 has a long half-life and can remain detectable several weeks after completed malaria treatment (**79, 80**).

Plasmodium falciparum histidine reach protein 2 (PfHRP-2) is expressed in gametocytes and all blood stages of *P. falciparum*. As this protein is released when the schizonts rupture, HRP-2 based RDTs can detect the sequestrated parasites that may escape the detection by microscopy. This can happen during pregnancy when malaria parasite sequester in placental tissues and avoid detection by microscopy (**81**).

pLDH: LDH is an enzyme and marker of different tissue damages and illnesses. pLDH is expressed massively by malaria parasites as it is produced in the glycolytic cycle of the asexual stage of all species of *plasmodium* (**82**), however pLDH's isoform can be differentiated from human LDH (**83**) and, pLDH-based RDTs can distinguish between *falciparum* and *non-falciparum* malaria infections. Moreover, pLDH is cleared from the blood stream after completed treatment much earlier than HRP-2 (**84**).

Aldolase-pan malaria antigen: Aldolase is "a major enzyme involved in the glycolytic cycle of *Plasmodium* and is released into the blood during infection"(**85**). Aldolase is a pan-specific antigen for *Plasmodium* detection since a large part of its amino acid sequences is relatively preserved in all *Plasmodium* species (**85, 86**). As the sensitivity of Aldolase based RDTs has shown to be low and dependent on parasite density (**81**), commercial use of Aldolase-based RDTs is not common.

1.7.2.3 Test principle of RDT

RDT is an immunochromatic test that detects malaria specific antigens. RDT is conducted by application of a few drops of capillary or venous blood on the RDT device. The labelled antibodies prepared against malaria antigen target is mixed with the blood. A few drops of lysing buffer are added to the sample for antigen release and antibody recognition. The testing process relies on the migration of liquid (blood and buffer) across the surface of a

nitrocellulose strip in the cassette test device. A capture antibody is bound to the nitrocellulose strip. If malaria antigen is present the labelled antibody-antigen complex will migrate along the nitrocellulose strip until it reaches the bound capture ab. The capture antibody binds to the labelled antibody-antigen complex and produces a visible line. In order to control the correct performance of the test i.e. to control the test for migration, another antibody capture specific for another epitope of the labelled abs is applied to the nitrocellulose strip for formation of the control line (**87**). RDT is able to detect the malaria parasite within 15-30 minutes.

Recently RDTs with combination of HRP-2 and pLDH enabling detection of both *P. falciparum* and other malaria species (*P. vivax, P. ovale and P. malariae*) has become available in some regions. However, these RDTs still cannot determine the exact species of non-*falciparum* malaria parasites.

RDTs are simple and user friendly and the device is tolerant for major different climatic conditions. An RDT device weighs only a few grams making it easy to transport and suitable for use in the field in different settings. RDTs are more expensive (per test) than microscopy. Yet, whilst microscopy requires good microscopes and qualified microscopists, blood staining facilities and electricity, the use of RDT requires none of these. Furthermore, RDTs have shown to be cost-effective since they improve management and health outcomes for non-malarial febrile patients as well as reduce the costs associated with prescription of expensive ACTs to RDT negative patients (**56, 88, 89**).

The significance of the RDTs in malaria surveillance has grown recently as the DNA from malaria parasites can be reliably extracted (See below). This can be used for molecular analysis such as detection of low level of parasitaemia below the detection limits of RDT and BS microscopy as well as drug resistance monitoring directly from RDTs stored during several months in tropical climate (**90**).

RDT has some disadvantages: it cannot determine parasite densities. Further, the HRP-2 based test remains positive several days, or even weeks after completed antimalarial treatment that prevents assessment of the clinical outcome of the treatment (**91**). Other issues such as RDT performance in the field situation as well as health workers' adherence to the test result have been studied and debated for a while. There are concerns regarding the safety of withholding antimalarial medicines to patients with negative RDT result. Depending on different epidemiological settings and health systems, the findings and conclusions are different and sometimes contradictory to each other (**54-56, 76, 92-94**). Nevertheless, ACTs are expensive and as mentioned earlier in settings with no access to microscopy facilities, presumptive treatment with ACT may prevent correct diagnosis for other and potentially deadly diseases. RDTs improve targeting of antimalarial treatment to malaria patients and may facilitate a more accurate case management of other febrile diseases (**95**). There is also the risk inducing the development of anti-malarial drug resistance with devastating effect on global malaria control efforts through overuse of the ACTs (**96-98**). After initial recommendation of use of RDTs for malaria diagnosis in settings where BS microscopy is not available (**66)** WHO started its new initiative called T3: *Test. Treat. Track*, in 2012 calling on the global malaria community to scale up diagnostic testing, treatment and surveillance for malaria in which RDT would play a significant role (**99**).

1.7.3 Molecular based diagnostic methods

As described earlier, the detection limits of both BS microscopy and RDTs do not allow detection of low parasitaemia carried by asymptomatic individuals in malaria endemic areas but these persons are still a potential reservoir for malaria transmission (**100**). Different molecular based diagnostic methods with high sensitivity for detection of very low parasitaemias are described below

1.7.3.1 DNA extraction

In order to detect malaria parasites with molecular methods, DNA is extracted from either fresh blood or blood dried on RDT (during the testing) or filter paper (FP). There are several methods for DNA extraction. The most common method when using small quantities of blood is Chelex-100, a cheap method which stability for further analyses decreases the longer storage time is (**101**). Simple elusion method is similar to Chelex-100. This is a more rapid method but is in comparison to Chelex-100 less stable (**102**). Column based methods (e.g. Qiagen or ABI) are used for larger volumes of DNA and for long DNA fragments. This method provides a better and more stable DNA quality but is more expensive and more laborious than the other two methods (**246**).

1.7.3.2 Polymerase Chain reaction (PCR)

The principle of the PCR technique is amplification i.e. converting very low quantities of DNA into very high quantities. The first report about the original method was published in 1985 (**103**). PCR technique relies on so-called thermal cycling i.e. heating and cooling. Each PCR cycle consists of three main steps and takes few minutes to complete. These steps are:

Denaturation: The biological material containing the original DNA sequence to be replicated is heated rapidly to 94°-96° C in order to melt the DNA sequence and separate the double stranded DNA into single-stranded DNA.

Annealing: The temperature is suddenly cooled down to ca. 68° C which enables the specific primer (a short DNA fragment complementary to the target DNA) to bind to the complementary DNA sequence of the single-stranded DNA template. *Taq*-polymerase (an enzyme responsible for building the DNA molecule by joining the nucleotides together creating a mirror image of the template) is attached to the double stranded DNA that is a formation of the primer and its complementary DNA sequence.

Elongation: The temperature is raised again to ca. 72° C and DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding deoxynucleotide triphosphates (dNTP). At the end of the cycle the temperature will rise again to 94°-96° C again for denaturation of the newly formed double stranded DNA and a new cycle will start (**104, 247**).

Real-time PCR or Quantitative PCR (qPCR) is a type of PCR-method enabling quantification of the desired product at any point in the amplification process by measuring the emitted fluorescence. Fluorescence is "the emission of light by a substance that has absorbed light or other electromagnetic radiation" (**Wikipedia**). The increase of the emitted fluorescence during each PCR cycle is proportional to the amount of PCR product. The levels of

fluorescence are measured at each PCR cycle by a detector. This method is called "real time PCR" since it measures the amplification as it occurs. This is assessed after every PCR cycle by measuring the quantity of fluorescence emitted while each double stranded DNA is produced. When the fluorescence is plotted against the number of cycles on a logarithmic scale, a threshold for detection of DNA-based fluorescence is set just above the background. The number of cycles at which the fluorescence exceeds the threshold is called the cycle threshold (C_t) (**104, 105**)

One of the earliest methods of nested PCR for parasite detection was designing primers targeting the small subunit ribosomal RNA (18S(r) RNA) genes of the four major human *Plasmodia* species i.e. *P. falcipaurm, P. ovale, P. malariae and P. vivax* (**106**). Later, the more sensitive method of targeting Cytochome b (Cyt b) gene in the mitochondrial DNA of all human *Plasmodium* species was introduced (**107**).

The detection limit of the PCR is between 0.05-10 parasite/µl, making it the most sensitive method for malaria parasite detection (**108, 109**). Moreover, PCR can identify all different types of human *plasmodium* species and mixed infections as well as quantify parasite rate. It can be used for screening of the entire population of large communities since DNA extraction can be done from blood stored on FP and RDTs (**90, 110**). However, PCR is expensive, requires advanced laboratory equipment, electricity and skilled personnel and therefore not possible to be used for routine malaria case management. PCR is mostly used for quality control of BS and RDT, monitoring of the malaria control programs as well as in research on the development of anti-drug resistance in parasites and drug efficacy trials (**111**).

1.7.3.3 Loop mediated isothermal amplification

More cost-effective molecular methods with lower demand on technical skills and advanced laboratory techniques are sought and research is on going. One method is the use of Loop Mediated Isothermal Amplification (LAMP), which can mass-amplify a few copies of DNA in a relative short time and under isothermal condition (**248**) It has shown promising results for the possibility of deployment of molecular techniques that can improve the diagnosis as well as surveillance of low-density parasitaemia in malaria elimination settings (**112,113**). However, parasite quantification cannot be done by LAMP.

1.7.4 Serology

This is another method of malaria diagnosis based on detection of antibodies against erythrocytic stages of malaria parasites. Serological markers can be used for evaluation of malaria transmission intensity and assessment of annual burden of malaria transmission as well as analysis of long-term trends in malaria transmission within and between communities in the same area (**114**).

Serology detects malaria parasite antibodies that are produced within two weeks after the initial infection and cannot differentiate between current or cured malaria infections. Thus, this method is not useful for clinical malaria diagnosis but is used for epidemiological studies as well as screening of asymptomatic infections among blood donors.

1.8 ANTIMALARIAL DRUGS

Things have definitely changed in malaria treatment since the time of the Romans when Sammonicus, who was the personal physician of the Roman emperor Caracalla, instructed his patients who had malaria to wear an amulet with the inscription "Abracadabra" meaning it came to pass as it was spoken **(115)**! But the path has been too long and the search for the perfect drug is still not completed.

Throughout history, there have been numerous traditional medicines for malaria treatment. Two important medicines used today (quinine and artemisinin) have both originated from traditional herbal medicine. As malaria has been a much more serious threat to poor countries in the tropical and subtropical climate zones, the development of the antimalarial drugs has not been in relation to malaria's impact on human life. The industry, drug companies and researchers' efforts to develop effective antimalarial drugs have been mostly formed by the need of soldiers involved in wars fought in malaria endemic regions and not patients living in those countries.

Jesuit missionaries in South America learned the use of Cinchona bark for malaria treatment from Native Incan herbalists. This was later named as Peruvian bark or Jesuit powder. The name Quinine originated from Incan word Quina-quina that means "holy bark". In 1820 the active alkaloids from the bark was isolated and named by French scientists Pelletier and Caventou. Quinine was used for malaria prophylaxis from mid 19th century. In order to meet the demands for quinine, large areas in Southeast Asia, particularly in Indonesia were made available for cultivation of Cinchona tree. Access to quinine contributed to the colonization of Sub-Saharan Africa, where malaria was a natural barrier for European colonizer to enter.

When Japanese troops controlled large territories in Southeast Asia during World War II, the allies' access to quinine reduced significantly. With limited access to quinine, thousands of soldiers stationed in Africa and the Pacific died from malaria.

In 1934, a new synthetic antimalarial drug named chloroquine was developed in Germany. Initially, it was ignored for a long time as it was considered to be toxic to human beings. After World War II, chloroquine was approved in the USA and used as antimalarial drug for decades to come (**116**). Despite the observation of the first signs of resistance against Chloroquin in late 1950s in some areas in Thailand and some remote areas in Venezuela and Colombia (**117**) and later development of widespread resistance against the drug globally, it remained as the drug of choice in large parts of Africa since alternative medicines were not available. Today, the use of chloroquine for uncomplicated *P. falciparum* treatment is not recommended unless in areas where resistance has not developed yet as well as for treatment of *P. vivax* (in combination with primaquine), *P. ovale* and *P. malariae.* For treatment of chloroquine-resistant *P. vivax*, ACTs combined with primaquine is recommended **(66)**.

The first document on the use of *Artemisia annua* for malaria treatment is from the mid 4th century (**118**) but it was during the Vietnam War and by order of Chairman Mao, that a group of Chinese researchers started the project 523 to find a sufficient treatment for malaria in 1967. The meeting to discuss short and long term goals for development of antimalarial drugs was held on May 23rd and thus the name "project 523". Extracted artemisinin from the

Artemisia annua showed to be a useful antimalarial drug. Combination therapy with other substances that together with artemisinin could enhance the antimalarial effect of the drug was suggested in early 1980s. The so called open door policy initiated by chair man Deng Xiaoping in the late 1970s (**249**), facilitated collaboration between China and commercial drug companies and resulted in manufacturing of ACTs in late 1990s (**119**, **120**). In 2001 ACTs were presented in WHO's list of essential drugs (**259**). Since 2006 ACTs are recommended as the drug of choice for first line treatment of uncomplicated malaria by WHO (**66**).

1.8.1 Rational behind combination therapy

Similar to some other diseases such as Tuberculosis or AIDS, mono-therapy for malaria can end up in development of drug resistance. Using the active metabolite of artemisinin compounds i.e. (artesunate, artemether, dihydroartesunate) with a partner drug showed to be effective for malaria treatment especially after development of global resistance against chloroquine (**23,121**).

Artemisinin and its derivatives such as artesunate or artemether are the most robust and rapid acting of all antimalarial drugs. Their capacity to reduce the infecting malaria parasite biomass is 10-100 times higher than other antimalarial drugs (**122,123**). Combination of artemisinin and its derivatives with a partner drug such as Lumefantrine or Mefloquine enables the rapid-acting artesunate or artemether to reduce parasite biomass quickly, whilst the slow-acting residual partner drug kills the remaining parasites that escaped from artemesinin. Moreover, the combination therapy may delay the development of drug resistance (**124-126**).

The main five common combination therapies recommended by WHO for treatment of uncomplicated *P. falciparum* malaria are: (artemether plus lumefantrine, artesunate plus amodiaquine, artesunate plus mefloquine, artesunate plus sulfadoxine-pyrimethamine and dihydroartemisinin plus piperaquine) (**66**)

1.8.2 The development of antimalarial drug resistance in *P. falciparum*

Genetic alterations expressed as single nucleotide polymorphisms (SNPs) associated with development of drug resistance in a parasite population are used for molecular surveillance and assessment of malaria drug resistance (**127, 128)**. Some of the genes found to be involved in drug resistance include:

P. falciparum Chloroquine resistance transporter gene (*pfcrt*), *P. falciparum* multidrug resistance 1 gene (*pfmdr1*) and *P. falciparum* multidrug resistance protein 1 (*pfmrp1*) (**129**).

P. falciparum Chloroquine resistance transporter gene (*pfcrt*): SNPs in this gene are showed to cause a significant reduction in the accumulation of the chloroquine in the digestive vacuole of the *Plasmodium* parasite where chloroquine exerts most of its effect (**130**). Additionally, it has been shown that *pfcrt* may be involved in resistance to other quinolones (**131**).

P. falciparum multidrug resistance 1 gene (*pfmdr1*): SNPs in this gene are associated with multidrug resistant phenotypes in *Plasmodium* parasite with regards to drugs such as mefloquine and artesunate (**122, 132**).

P. falciparum multidrug resistance protein 1 (*pfmrp1*): SNPs in this gene have also been shown to be associated with response to antimalarial drugs (**133**).

As mentioned earlier, about a decade after deployment of chloroquine as the first established malaria chemotherapy, signs of resistance were observed in Southeast Asia and later South America. When new drugs were introduced later in the 60s and 70s, malaria parasites managed to develop resistance against them, too. After initial reports on prolonged parasite-clearance times from the Thai-Cambodian border (**96, 134, 135**), *P. falciparum* resistance against artemisinin across mainland Southeast Asia is now reported (**135**).

Recently, an increased prevalence of genetic markers associated with artemether-lumefantrine tolerance or resistance in Africa after deployment of artemether-lumefantrine as first-line treatment for uncomplicated malaria was observed (**136**).

1.9 VECTOR CONTROL MEASURES

Although the clear connection between mosquito and malaria transmission was first discovered in late 19th century, malaria control has historically been done through environmental measures focusing on vector control. The initially successful malaria eradication attempt in the mid 50s and 60s, which resulted in malaria elimination in many countries around the world, was achieved mainly by vector control (**137**).

Current methods used for vector control around the world include chemical methods such as residual insecticides and mosquito nets, environmental methods such as drainage and biological methods such as use of Larvivorous fish (**138**,**139**).

Chemical methods are the most commonly used methods of malaria control are chemical methods such as residual insecticides and mosquito nets.

1.9.1 Residual insecticide

The principle of using insecticide residual spraying (IRS) is based on the *Anopheles*' feeding and resting behaviour. After taking its blood meal, the *Anopheles* lands on a closest indoor surface to rest and digest the blood meal for several hours. If the indoor surface is treated with a residual insecticide, the *Anopheles* will die before it is capable to transmit malaria. The most known insecticide, Dichlordiphenyltrichloroethane (**DDT)** was synthesized in 1870's but its insecticidal effect was first discovered by a Swiss scientist H. Müller in 1939 (**140**). During the WWII, the allies used DDT mostly against Typhus. Later in the 40's and 50's it was used on a broad scale to fight malaria mosquitoes preliminary in Europe and USA. The campaign resulted in great success in Europe and North America but showed to be much less successful when used in areas with stable malaria transmission (See below) (**141**).

1.9.2 Mosquito net

Use of bed-nets for protection against mosquito biting is documented as early as 2700 BC in China (**1**). Insecticide treated nets (ITN) were developed in the1980's for vector control and launched by WHO in late 1990s (**142, 143).**

Numerous studies have shown a large protective impact of ITNs and effect on malaria transmission. ITNs have shown to reduce malaria morbidity by 25% to 50% and to reduce child mortality by 20% (**144, 145**). It has also been shown that ITNs are providing protection to everyone in the community with high ITN coverage as it reduces the number of infected mosquitoes in general (**146**).

In recent years, the use of Long-lasting insecticidal nets (LLIN) is becoming more common. LLINs are pre-treated with insecticide upon the manufacturing and the insecticidal effect lasts for 4-5 years, hence no re-treatment is needed (**147**).

Significant reduction of malaria burden following intervention with IRS in recent years has been reported (**13**). However combination of both IRS and LLIN has shown to be even more effective (**148**). Moreover, use of different types of insecticides in combined interventions may improve management of vector resistance against the insecticides (**149**).

1.9.3 Vector development of resistance against insecticides

The *Anopheles* mosquito has shown to be able to adapt to different environments. It has been effective in developing resistance/tolerance against insecticides and changing its feeding and resting behaviour (**237, 250**). *Anopheles* resistance against DDT was widespread globally just a few years after deployment of DDT for vector control (**150**).

In addition to DDT, other more commonly used insecticides for vector control are: active component of pyrethrum flowers (pyrethrins) or its synthetic form (pyrethoroids) and carbamates as well as organochlorines and organophosphates. The most commonly used insecticides for vector control used in LLIN and some IRS are pyrethroids.

Several recent reports from different parts of Africa have shown changes in the vector population towards presence of outdoor-active and early-feeding *Anopheles* population (i.e. a decline of indoor active *An. gambiea s.s.* and an increase of the outdoor active *An. arabiences*). This highlights the risk of failure of current used vector control interventions (mosquito nets that focus on indoor biting mosquitoes) (**151- 155**).

Unfortunately with increasing vector resistance against pyrethroids and other commonly used insecticides, the long-term effectiveness of LLIN and IRS is in jeopardy. This highlights the urgent need for developing more effective vector control measures (**156-158**).

Figure 6: John Singer Sargent (1856-1925), *Mosquito Nets,* 1908, American. Oil on canvas.57.1 × 71.7 cm. Courtesy of the Detroit Institute of Arts (http://www.dia.org/), Detroit, Michigan; Founders Society Purchase, R. H. Tannahill Foundation fund, 1993.18/The Bridgeman Art Library, New York, New York (159).

2 MALARIA CONTROL, ELIMINATION AND ERADICATION

Malaria control is defined as reducing malaria morbidity and mortality to a locally acceptable level through use of current available tools for prevent and cure of the disease. Malaria elimination refers to "reducing the incidence of locally acquired malaria infection in a specific geographic area to zero" while malaria eradication is defined as "reducing the global incidence of malaria to zero" (**18**). After child immunization, malaria control is the most cost-effective health intervention. The cost for malaria elimination however, is much higher (**18, 160**).

2.1 INITIAL SUCCESS AND FAILURE

WHO initiated Global Malaria Eradication Program (GMEP) in 1955. The main tools to achieve malaria eradication were use of DDT for vector control and chloroquine for malaria treatment (**141**).

Whilst the main vector in temperate zones with seasonal malaria transmission and cold winters are more zoophilic species of *Anopheles*, the dominant *Anopheles* species in in Sub-Saharan Africa are strongly anthrophilic (**161**). By the end of 19th century and early 20th century, malaria had already started to decline in Europe and North America. Improved living conditions that reduced the contact between humans and the vector in combination with seasonality of malaria transmission as well as improved public health are believed to be the main factors behind the spontaneous decline in malaria in these parts of the world. Implementation of DDT was a successful contribution that resulted in elimination of malaria in almost all Europe and North America in 1950s (**162**).

The attempt to eliminate malaria in the rest of the world especially in Africa ended in failure mainly due to lack of efficient infrastructure, development of vector resistance to DDT and later parasite resistance to chloroquine in combination with dominance of anthrophilic *Anopheles* species and fatal *P. falciparum* malaria. In 1969, GMEP was eventually abandoned (**141**).

2.2 NEW GLOBAL STRATEGY AND CHALLENGES A HEAD

The years of 1970s and 1980s were the dark period in modern history of malaria control. In addition to above mentioned factors, economical crisis in the beginning of 70's, political instability, operational difficulties and lack of field-related research were among the factors contributing to the deterioration of the situation. In 1998, the Roll Back Malaria (RBM) partnership was launched as an effort to provide a coordinated global response to malaria transmission (**163**). The new strategy of malaria control, which replaced the previous eradication strategy, were introduced as:

- "Control of malaria to reduce the current burden and sustain control as long as necessary
- Eliminate malaria over time country by country and
- Research on new tools and approaches to support global control and elimination efforts" **(164).**

In 2000, the head of African states set out to "halve malaria mortality by 2010" through implementation of RBM's malaria control strategy **(165, 142).** Further, fighting malaria was made one of the United Nations' millennium development goals (MDG). However the direct and indirect association of malaria with child mortality, maternal health and poverty, makes it an equally important element of reaching the other MDGs, too **(166)**.

In 2005, the global goal was set to "ensure that at least 80% of those at risk of, or suffering from, malaria benefit from major preventive and curative interventions by 2010. This would reduce the burden of malaria by at least 50% by 2010 and 75% by 2015" (167).

WHO updated these targets in 2011 to the following:

- "Reduce global malaria deaths from 2000 levels by 50% in 2010 and to near zero preventable deaths in 2015.
- Reduce global malaria cases by 75% by end 2015 (from 2000 levels).
- Eliminate malaria in 8-10 countries by 2015 and afterwards in all countries in the pre-elimination phase today".

In a long-term perspective, malaria eradication would be reachable by "reducing the global incidence to zero through progressive elimination in countries" (168).

However, in 2013 less than 50% of the population in sub-Saharan Africa had access to an ITN in their household (15).

2.3 FROM CONTROL TO ELIMINATION

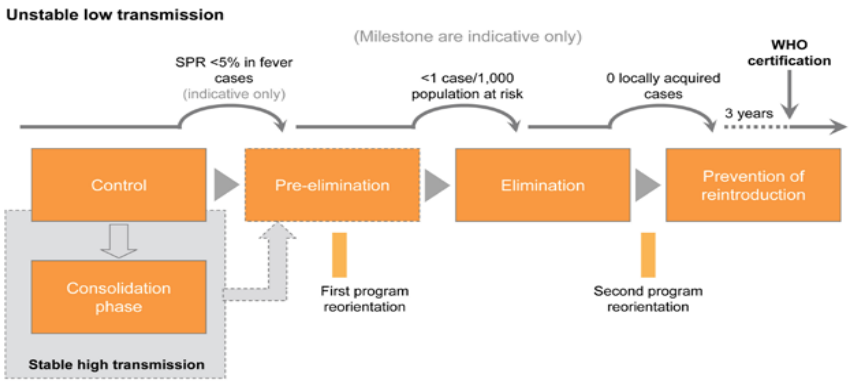
Malaria elimination requires three fundamental elements: prevention, treatment and surveillance **(**169). Moving from malaria control to elimination goes via pre-elimination. It is a transmission state in which the slide (or RDT) positivity rate (SPR) of all febrile patients with suspected malaria is <5% or when malaria incidence is <5/ 1000 people at risk in a specific region. This requires scale up of malaria control measures as well as general enhancement of the health system to reach a sustainable malaria control i.e. maintain and gradually improvem the achieved low malaria transmission status (**170,165**).

A crucial task in this process is how to avoid malaria resurgence defined as "the reappearance of new infection in significant numbers after reduction of malaria transmission by malaria control efforts" (**171,172**). Between 1930-2000 more than 70 resurgence events in 63 countries were observed of which most of them were associated with weakness of the malaria control programs (**172**).

The process of moving from malaria control to pre-elimination and later elimination requires reorientation of the activities of the national malaria control program. This includes reduction of the parasite reservoir through early diagnosis and treatment with effective medicines. Further, health information system needs to be strengthened to ensure a sustainable access to malaria control measures, entomological monitoring as well as management of the imported malaria cases (**169, 173**). Surveillance, in particular, is considered to be a main component of any program aiming to interrupt malaria transmission completely (**174**) (See figure 7). Who gets malaria, where and when the malaria transmission occurs in any specific transmission setting are of great importance for a successful approach towards malaria elimination.

Coordinating research to obtain knowledge on the on-going changes in epidemiological trends of malaria is critical to move this task forward (**175**). In addition, a continuous trend of malaria reduction requires commitments of both donors and the communities, a well-prepared plan to prioritize the limited resources as well as a functioning global cooperation (**176, 177**).

Figure 7: From control to elimination. Source: WHO, Global action plan for a malaria free world (18)



2.3.1 Effective coverage

The effective coverage i.e. "the proportion of the population in need of an intervention who are using an effective intervention that is affected by both access and adherence to the intervention" (**178-180**) is an important factor in the process of reaching malaria elimination.

Feasibility of this process requires development of effective tools accessible to and adhered by the population at risk. Adherence to the control measures depends on social, cultural and economical factors. Therefore delivery of the control measures to the population at risk must be combined with provision of information to and training of both community members as well as the health workers. There are various factors influencing the accessibility to malaria control measures such as the health system in general and its infrastructure in the region as well as current distribution strategies of the control tools. The distribution of the control measures is often not equitable. It has been shown that 20% of the poorest people at risk of malaria carry 58% of the total malaria burden and they tend to have less access to the control measures (**178, 181, 182**).

3 ZANZIBAR

Figure 8: Map of Tanzania and Zanzibar

Source: Wikipedia (251)

All studies included in this thesis were conducted in Zanzibar, Tanzania. Zanzibar archipelago is located ca. 50 kilometres off the coast of Tanzanian mainland. This semi-autonomous region consists of two main islands of Unguja and Pemba covering and area of 2,461 km^2 (**183**).

3.1 CLIMATE

Located just south of the Equator, the climate of Zanzibar is tropical and humid. The seasons are defined mostly according to rainfall, one long rain period in April-June and one short rain period in October-November. The period between July and September is the dry, cool season while the period between December and March is the dry, hot season.

The total population of Zanzibar in 2012 was 1.3 million of whom the majority (60%) is rural and 64% of the population live in Unguja. The population density in Zanzibar is 530 per km^2

and the life expectancy at birth is estimated to 60 years. The literacy rate is around 70%. The annual growth rate in Zanzibar is 2.8% and children make 13% of the total population. HIV prevalence in Zanzibar is around 1% (**184).**

For years, Zanzibar was the centre for the Swahili culture. Swahili, originated from the Arabic word "Sahil", the coast, is the language spoken in Zanzibar and in the costal regions is East Africa from south Somalia to north Mozambique as well as in Kenya, Tanzania, Uganda, Rwanda, Burundi and Eastern parts of the Democratic Republic of Congo. Often called the spice island, Zanzibar is one of the main locations for clove production. Zanzibar was a Sultanate for a long period. The name Zanzibar comes from the Arabic word of (Zinjibar =the land of the slaves) as for a long period of time, until late 19th century, Zanzibar was one of the several centres for slave trade in and around Africa. Between late 19th century and 1964, Zanzibar was a British protectorate but after the 1964 revolution, Zanzibar joined with the Tanganyika republic to form the United Republic of Tanzania. Zanzibar has its own government and parliament and is responsible for all non-union issues such as health care system.

Zanzibar consists of ten districts (four in Pemba island and six in Unguja Island). Each district is divided to constituencies and then subdivided into shehias that are the smallest administrative unit in Zanzibar (**185**). Subsistence farming, especially food crops, is the main source of income to most of the inhabitants. Fishing and tourism contribute to the people's income. In recent years the real gross domestic product has grown (to ca. 7.0% in 2012). Yet, Zanzibar is still a low-income region. In 2012 the per capita income was US$ 638 per year i.e. <2 USD/day (**186**).

3.2 HEALTH SYSTEM IN ZANZIBAR

The health system in Zanzibar is divided between private and public health care systems. Private health care facilities and hospitals are mainly concentrated in the urban parts of Zanzibar (mainly Unguja). Major health services are however provided by the public health sector. Three levels of public health care facilities in Zanzibar are:

- Primary level: Divided into primary health care units and centres (PHCUs and PHCCs)
- Secondary level: Four district hospitals (all located in Pemba)
- Tertiary level: The main referral hospital in Stone town (Unguja)

Throughout Zanzibar, there are 134 PHCUs providing basic outpatient care with no laboratory services. Malaria is mainly diagnosed with RDT. PHCCs include basic laboratory services such as microscopy for malaria diagnosis as well as in-patient care. More than 95% of the population of Zanzibar live within five-kilometre distance from a public health facility (**187**). The under-five child mortality rate has decreased from 141 per 1000 live births in 2002 to 73 in 2010 (**188, 189**). Improved breastfeeding practices, IMCI and high immunization coverage as well as the recent years' dramatic reduction of malaria incidence are the main reasons for this significant reduction of the under-five child mortality (**212, 252**).

3.3 MALARIA IN ZANZIBAR

Malaria has for a long time been one of the major burdens on the health system of Zanzibar. Until recently, Zanzibar was considered to be a typically malaria high endemic area. WHO started an eradication program in the late 1950's. Malaria eradication in Zanzibar was not achieved but after reorganization of malaria eradication program in 1967, it succeeded in reducing the malaria prevalence significantly. In 1968, malaria was not considered a health problem in Zanzibar and the program was subsequently abandoned. Malaria prevalence in Zanzibar increased immediately following the development of *P. falciparum* resistance to chloroquine, vector resistance to DDT and technical and logistical difficulties. By the mid 1990's one third of all deaths at the hospitals (approximately 3300 deaths annually) were attributed to malaria (**190, 191**). In 2001 malaria accounted for 41% of all diagnosis in health facilities and was ranked number one cause of morbidity and mortality in Zanzibar (**253**).

After launching the combined malaria control strategies by RBM and WHO, Zanzibar started its attempt to control malaria in the beginning of the 21st century through deployment of control measures shown in Table 1.

Zanzibar was among the first regions in Africa that deployed ACT for malaria treatment. The Zanzibar Ministry of Health and Social Welfare (after 2010 the ministry of health became an independent ministry (MoH)) changed its malaria treatment guidelines and deployed ACT as first line therapy instead of chloroquine in 2002. This policy was implemented in September 2003. Short afterwards large-scale distribution of ITN and later LLIN as well as biannual IRS treatment of interior walls of the houses of Zanzibar started. The impact of deployment of ACT and LLIN resulted in a significant reduction of malaria morbidity and mortality within three years of deployment of ACTs and later LLIN (**3, 192, 193**).

Paracheck® RDT for *P. falciparum* malaria diagnosis was first introduced in some parts of Zanzibar by the non-governmental organisation Médecins Sans Frontières (MSF) in the mid 2000s (**56**). By 2007, RDT was deployed in all PHCUs where BS microscopy was not available. In 2008 the diagnostic criteria for malaria was changed to confirmation by either RDT or BS microscopy. The previously widely used diagnostic criteria of so-called "clinical malaria" were removed from the health management information system (**192, 193**). Malaria RDT was in 2009 incorporated in the Zanzibar version of (IMCI) guidelines **(193)**. Later, in 2011, Paracheck® RDT was replaced with SD-Bioline Malaria Ag P.f/Pan which enables detection of other malaria species than *P. falciparum* alone.

In 2008, the Malaria Early Epidemic Detection System (MEEDS), a system for reporting malaria cases electronically through mobile phones on weekly basis was established. Following further decline of malaria cases, Malaria Case Notification (MCN), for follow-up of all malaria cases, testing and treatment of household contacts and provision of preventive interventions and health information was introduced in 2012 (**194**).

By 2012, the parasitologically confirmed (with BS microscopy or RDT) malaria positivity rate in all age groups was <1% and in August 2013, Zanzibar Malaria Control Programme (ZMCP) changed its name to Zanzibar Malaria Elimination Programme (ZAMEP) marking the entrance of the new phase of approaching malaria elimination in Zanzibar.

Table 1: malaria control interventions in Zanzibar 2002-2012

Year	Interventions
2002	Introduction of ACT as the first line malaria treatment at all public health care facilities in Zanzibar.
2003	Implementation of the ACT at all public health centres in Zanzibar, free of charge, in September 2003.
	1st line treatment: Amodiaquine + Artesunate (Aq-As).
	2nd line treatment: Artemether – Lumefantrine (Co-artem®).
	3rd line treatment: Quinine and the drug of choice for severe malaria.
	(SP) for (IPT) in pregnancy as well as uncomplicated malaria in pregnant women.
2004	Initial distribution of ITNs by ZMCP and through schools and community leaders, free of charge.
2005	Overall distribution of LLIN to children under five and pregnant women.
2006	First round of IRS in July 2006.
	Implementation of RDT at some peripheral health centres.
2007	Wide scale use of RDT at the peripheral health facilities for malaria diagnosis. 2nd and 3rd rounds of IRS.
2008	**4th** round of IRS, (universal distribution of LLIN i.e. 2 nets per households).
	Introduction of MEEDS
2009	New treatment guidelines for second line malaria treatment (**222**).
	1st line treatment: Amodiaquine + Artesunate
	2nd line treatment: Quinine
	3rd line treatment: Parenteral Quinine
	SP for IPTp
	Uncomplicated malaria in the first trimester oral Quinine, in the second and third trimester (ACT).
2010	5th round of IRS
2011	Deployment of combined HRP-2 and pLDH (SD-Bioline Malaria Ag P.f/Pan) RDT for detection of both *P. falciparum* and mixed malaria infections.
2012	LLIN distribution (two nets per household)
	IRS policy change – from universal to focal targeting hotspots (carbamate)
	Introduction of MNC and household screening

4 RATIONAL FOR THE DOCTORAL PROJECT

This thesis consists of two parts. Part one (study I and II) describes the prospects of the feasibility of achieving malaria elimination in Zanzibar following the implementation of malaria control interventions and the assessment of the effective coverage of these intervention after the significant reduction of malaria transmission in Zanzibar.

Despite the recent years improvements, *P. falciparum* malaria still remains a major cause of death among small children in sub-Saharan Africa. Zanzibar has been among the first to implement the combined malaria control strategies by initiating deployment of ACT in 2003, mass distribution of LLINs and bi-annual IRS in 2006 and use of RDT for malaria diagnosis. After the initial reports of marked reduction of incidence of *P. falciparum* malaria the official goal of Zanzibar was set up to eliminate malaria on the isles.

A successful transition from control via pre-elimination to elimination of malaria depends on three important factors of prevention, treatment and surveillance. However, considering Zanzibar to be a previously high endemic area in sub-Saharan Africa, the potential and feasibility of such a transition is to be studied. Zanzibar's attempt to eliminate malaria provides unique research opportunities and challenges, particularly to assess the sustainability of the present achievement towards pre-elimination and further towards elimination as well as prevention of re-introduction (Study I).

Sustaining a high coverage of vector control interventions is important for maintaining the state of low malaria prevalence and for further decline in malaria transmission as well as prevention of malaria resurgence. Zanzibar provides a unique research opportunity for assessment of sustained high effective coverage of vector control measures following the significant decline of malaria transmission in recent years (Study II).

Part two (study III and IV) describes the efficiency of different malaria diagnosis tools in the new epidemiological context that emerged after rapid decline of malaria in Zanzibar including use of molecular tools for surveillance.

Good malaria case management includes an effective and quality assured system for malaria case detection that ensures improved targeting of ACTs to patients with malaria infection.

Overuse of the expensive ACTs will not only be a substantial financial burden on the health care system, but more importantly it will prevent other causes of fever (e.g. pneumonias which require antibiotics) from being appropriately treated.

The usefulness of RDTs in the new malaria endemic context in Zanzibar including its performance within IMCI algorithm for childhood illness management needs to be assessed particularly with regards to adherence to test results as well as sensitivity and specificity as compared with detection of malaria parasites with BS microscopy and PCR (see below Study III and IV).

We also investigate the field applicability of RDTs as a source of parasite DNA for DNA extraction for molecular surveillance. This underlines the potential of RDTs in modern malaria control, not only as a key diagnostic tool but also for molecular surveillance (Study IV).

5 AIMS AND OBJECTIVES

5.1 OVERALL AIM

To assess the effectiveness of malaria control tools and interventions for achieving malaria elimination in Zanzibar.

5.1.1 Specific objectives

- To assess the impact of combined malaria control interventions on malaria transmission in Zanzibar.

- To assess effective coverage of malaria preventive measures following the significant reduction of malaria burden in Zanzibar.

- To assess the efficiency of HRP-2 based RDT for *P. falciparum* case detection including its performance within the local version of the integrated management of childhood illness (IMCI) algorithm among febrile patients at first level health care facilities in the new context of low malaria transmission in Zanzibar and to evaluate the health workers' adherence to the RDT results.

- To compare and evaluate different methods of DNA extraction from RDTs for molecular surveillance.

6 STUDY SITES

The studies included in this PhD project were conducted in two districts of Micheweni (Pemba island) and North A (Unguja Island) in Zanzibar (figure 8 and 9). These two districts are with regards to their demographic composition as well as malaria epidemiology considered to be representative for Zanzibar overall. Both districts are mainly rural and have a population of about 100,000 each out of 1.3 million in Zanzibar. Both districts have access to one PHCC, which includes a hospital with out and inpatient care and laboratory equipment, including malaria microscopy service and blood transfusion facilities. Two private health facilities have been established in each district in recent years. *Anopheles gambiae* and *Anopheles funestus are* the main vectors.

Following the mass distribution of LLIN and universal deployment of IRS in all households throughout the isle for vector control and introduction of ACT for malaria treatment, a significant reduction of malaria morbidity and mortality was achieved (**3**). With increasing coverage of malaria control measures a further decline of parasitologically confirmed malaria positivity rate was observed (**187**).

Study III was a health facility based study, conducted in 12 primary health care facilities (one PHCC and five PHCU from both Micheweni and North A districts). Health facilities were selected with regards to their geographical distribution as well as the skilled cadre of conduct of the study. Zanzibar introduced RDTs for malaria diagnosis at PHCUs in 2006.

6.1 ETHICAL CONSIDERATION AND CLINICAL TRIAL REGISTRATION

All studies were conducted in line with the principles stated in the latest version of the Declaration of Helsinki and Good Clinical Practice (**254, 255**).

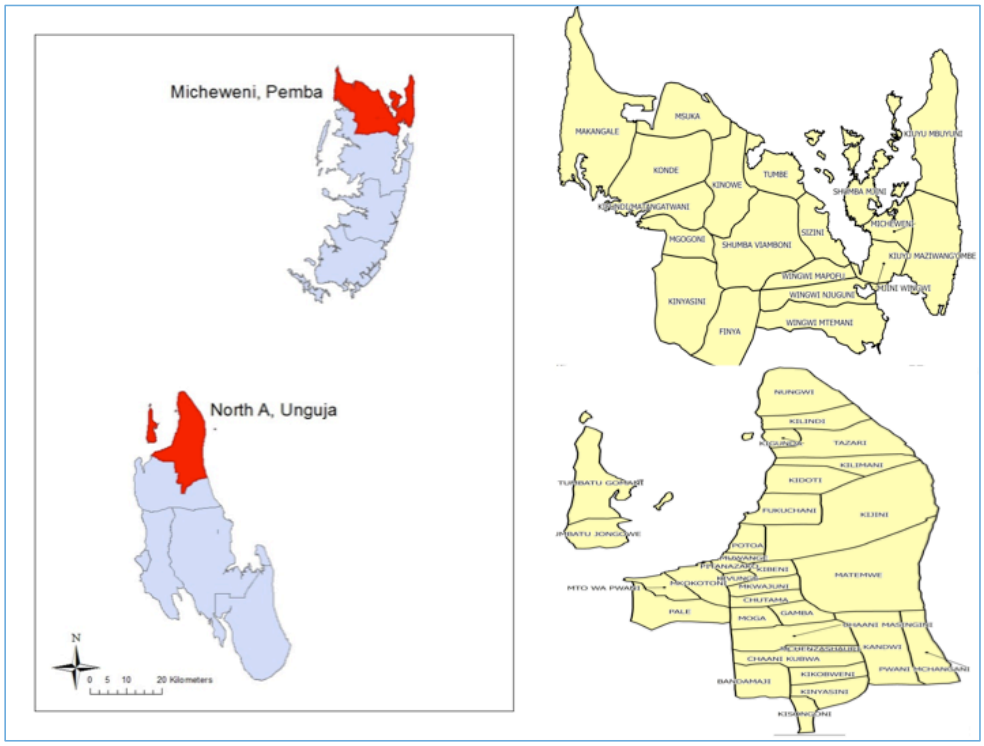
Studies conducted in Zanzibar were approved by the Zanzibar Medical ethics committee previously known as The Zanzibar research Council, reference numbers: (ZHRC /RAP0/03/2004, ZAMEC 0001/09, ZAMEC/ST/0001/010, ZAMREC/0001/JUNE 011, ZAMREC/0001/APRIL/013) and the Regional Ethics Review Board, Stockholm, Sweden (Reference number: 2009/387-31).

Ethical approval for molecular analysis conducted in Sweden was obtained from the Regional Ethics Review Board, Stockholm, Sweden, Reference number: (2009/387-31).

Informed written consent from all study participants were obtained prior to their participation in the study. The consent was witnessed. For children, a proxy-consent from parents or their legal caretaker was received. Information meeting together with local leaders (Shehas) were held prior to start of each cross-sectional survey in order to inform the communities about the anticipated study. Clinical Trial registration on ClinicalTrials.gov for study II and IV with study identifier NCT01002066 was made (not applicable for study I and II).

Figure 9: study sites and shehias distribution in Micheweni and North A districts, Zanzibar.

Source: ZMCP



7 PART ONE

7.1 MATERIALS AND METHODS

7.1.1 Study population sampling and data collection

Study I

This was a longitudinal, observational and multidisciplinary study conducted in both Micheweni and North A districts. Malariometric data including community parasite prevalence, health facility data, vital statistics and entomological data were obtained as following:.

Cross sectional surveys conduced between 2003 and 2012, which included eight surveys (2003, 2005-2009, 2011 and 2013). Sampling methods are described under study I. Health facility data from 1999 to 2013 were collected from all 26 PHCUs and PHCCs in both districts. Vital statistics for North A district for the period of 1998 to 2012, were obtained from the District Commissioner's Office in North A. A sub-study on risk factors for malaria infection included data collected in study III. Monthly rainfall data between 1999 and 2012 for North A and between 2005 and 2012 for Micheweni district were obtained from the official registers of the Zanzibar Office Division of the Tanzanian Meteorological Agency of the Ministry of Communication and Transport.

Entomological data were collected from several sites on Unguja and Pemba islands from 2005 onwards where mean human biting rates using the human-landing catch method were determined.

Study II

This study was conducted in two districts of Micheweni and North A. The sampling was done according to two-stage cluster sampling technique (**195**). Sampling units were the same randomly selected shehias used in the previous cross sectional studies. From each shehia households were randomly selected proportional to the size of the shehia. Assuming a proportion of 50% of children under five sleeping under bed-nets, and accounting for a cluster effect of two, a sample size of 192 children under five was needed to determine LLIN use with an absolute precision of ±10% and a 95% confidence interval (CI). Since the study was conducted in conjunction with the cross sectional survey of 2009, the total number of caretakers interviewed was 560 in total which was larger than required.

7.1.2 Laboratory and molecular methodologies

Study I

Molecular analysis included DNA extraction and PCR analysis. Two PCR methods followed by restriction fragment length polymorphism (RFLP) analysis were used: cytochrome b (cytb) nested PCR (**224**) and cytb SYBR Green qPCR (**Xu W *et al.*, unpublished**). Parasite density was estimated by qPCR. The molecular analyses are described further in part two under study III and IV

7.1.3 BS microscopy and RDT

In all cross sectional surveys conducted between 2003 and 2009, thick BS for malaria diagnosis were collected from all study participants. All slides were stained with 5% giemsa staining and experienced microscopists conducted microscopy examination for *P. falciparum* parasite detection as well as determination of parasite densities. If less than ten parasites were detected per 200 WBCs, examinations were extended to 500 WBCs. Blood slides were considered negative if no asexual parasites were found in 200 high-power fields. Asexual parasite densities were calculated against 200 WBCs assuming 8,000 WBCs per µl of blood (**70**). Quality control (independent second reading) was done for all positive slides as well as 10% of the negative slides. RDT replaced microscopy with a *P. falciparum* specific HRP-2-based device (Paracheck-pf®) in 2011 and in 2013 a combo RDT detecting both HRP-2 and pan-*Plasmodium* lactate dehydrogenase (SD-Bioline Malaria Ag P.f/Pan) was used.

7.1.4 Filter paper preparation

In cross sectional studies conducted from 2005 and onwards, approximately 100 µl of blood (3-4 blood drops) was collected from all consented participants on FP (3MM Whatman) and then dried and stored according to standard procedures. After completion of the studies, samples were transported to Karolinska Institutet in Stockholm, Sweden for further laboratory analysis such as PCR technique for assessment of potential reservoir of low malaria parasitaemias and determination of molecular markers for drug resistance (**127, 128, 196**) as well as malaria serology.

7.1.5 Malaria serology

The antimalarial antibody responses was assessed by enzyme-linked immunosorbent assay (ELISA) from blood spots collected during the cross sectional survey in 2009 on FPs as described above. The serology analysis was done as described previously (**114, 197**). The presence of immunoglobulin G antibodies against three *P. falciparum* blood stage proteins, apical membrane antigen-1, (PfAMA-1), glutamate rich protein (PfGLURP) and merozoite surface protein (PfMSP-1) was tested in all samples. Individuals were considered positive if they responded to one or more antigens.

Study II

Study II included no laboratory or molecular procedures.

7.1.6 Data management and analysis

Study I

Data entry and validation were done by Microsoft Access and Microsoft Excel. Statistical analyses for cross-sectional surveys, health facility records, vital statistics, and rainfall data were performed using STATA 12 &13 software. Pearson correlation coefficients [rp] were calculated to assess the linear relationships between monthly rainfall and outpatient confirmed malaria incidence and a Poisson regression model were used to assess the effect on malaria incidence and the interaction between age (categorized as <5 and >5 years of age) and calendar year.

Malaria serology data were analysed using sero-conversion rate (SCR) to estimate the force of infection i.e. “incident malaria cases per unit of population-time”. This was done by adjustment to a “simple reversible catalytic model” to the measured sero-prevalence, stratified into yearly age groups by using “maximum likelihood methods” (**198**). Temporal change in SCR was identified using profile likelihood plots and likelihood ratio tests against models with no change (**197, 199**).

Study II

Data entry was conducted using CSPro program, thereafter the data was transferred into Excel for data cleaning and coding of the open-ended questions. The data were then imported into STATA software where open-ended answers were coded into different categories (ex. Age and years of education were re-coded into categorical variables). Data analysis was conducted using STATA 10 software. These included univariate analysis (means and medians of continuous variables or frequencies and proportion of categorical variables), bivariate analysis (chi-square test or logistic regression ex. the factors associated the use of LLIN among children under five) and multivariate analysis (all variables that were at the significance level of $p \leq 0.25$ in the bivariate analysis). Adjusting the *p*-value for cluster effect on the shehia level was done. Equity analysis was done to determine the weights for an index of the asset variables (**200**). The study population was hence categorized into socio-economic quintiles based on an asset index based on type of floor, walls and roof, source of water and light, type of toilet and cooking facilities, and owning 20 different possessions such as bicycle or radio. Effective coverage of LLIN and IRS in children under five belonging to different socio-economic quintiles was then compared. The significance of the outcomes was analysed using logistic regression.

7.2 RESULTS

Study I

Table 2: Number of households Surveyed, age and sex of the cross sectional studies' participants in Micheweni and North A districts (2003, 2005-2009, 2011 and 2013)

a) Micheweni

Year	03	05	06	07	08	09	11	13
HH, n	550	397	397	448	3493	499	488	427
<5	476	283	323	395	19209	476	312	399
5-14	222	323	301	420	36083	222	417	820
>14	982	889	952	1180	ND	982	761	1191
Female %	45%	48 %	54 %	50 %	49 %	51 %	57 %	51%

b) North A

Year	03	05	06	07	08	09	11	13
HH, n	621	474	483	517	3477	498	528	401
<5	677	314	320	375	15735	281	358	333
5-14	601	441	386	387	28897	220	427	618
>14	1535	1189	1151	1124	ND	888	886	1073
Female %	51 %	48 %	50 %	51 %	50 %	52 %	60 %	54 %

7.2.1 Intervention uptake

After the mass distribution of LLIN in early 2006 the reported proportions of people sleeping under ITN/LLIN according to the cross sectional surveys conducted between 2006 and 2013 increased by 61% and 34% to 70% and 71% in Micheweni and North A, respectively.

However, children under five were significantly more likely to use ITN/LLIN (78%) than individuals ≥5 years (62%). From 2008 to 2012 the proportion of households reporting having had IRS within the last year was over 85% in both districts.

ACTs were available in all PHCCs without any documented stock-out period since their implementation in late 2003.

7.2.2 Community parasite prevalences and serology

Figure 10 shows the overall *P. falciparum* parasitaemia of all ages at the time of cross-sectional surveys in both districts of Micheweni and North A determined by BS microscopy or RDT and in relation to malaria control interventions.

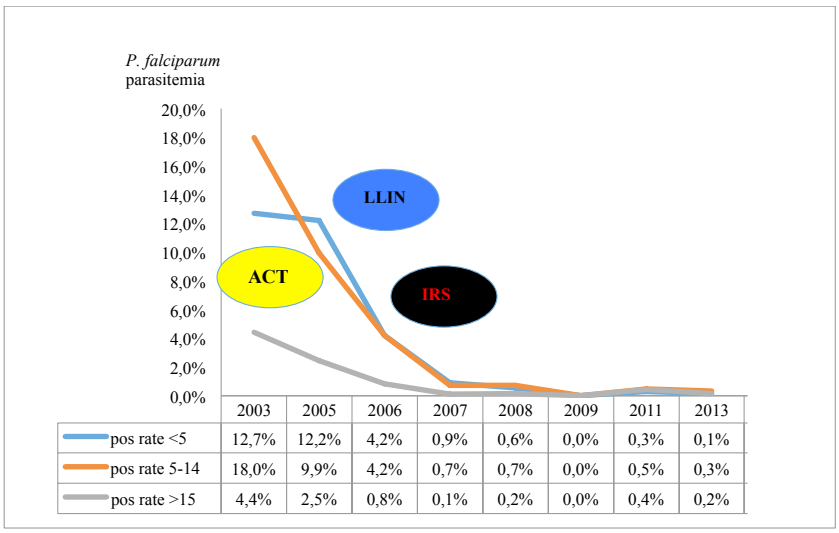
Between 2003 and 2013 a mean reduction of 97.0% of *P. falciparum* parasitaemia was observed. The main reduction in parasite prevalence occurred during 2006 and 2007 after the introduction of LLINs and IRS. Thereafter prevalences were maintained around 0.4% in Micheweni and 0.2% in North A.

In 2013, the PCR determined community parasite prevalences were 2.7% in Micheweni and 1.8% in North A (mean 2.3%, 95% CI 1.8-2.8). This represents a mean reduction of 89.0% as compared with 2005.

Malaria seroprevalence and force of infection

Serological analysis of samples from 2009 showed an increase in malaria seroprevalence with age. The overall seroprevalence was 37.4% (595/1598) in Micheweni and 19.3% (238/1232) in North A. Profile likelihood analysis identified distinct changes in SCRs at 5 years of age (i.e. after 2004) in both districts. SCR values indicated approximately 3-fold reduction in exposure in Micheweni and 5-fold reduction in North A comparing pre- and post-intervention initiation periods.

Figure 10: Malaria parasitaemia detected with BS microscopy or RDT in cross sectional surveys (2003-2013) in Micheweni and North A Districts combined, Zanzibar



	2003	2005	2006	2007	2008	2009	2011	2013
pos rate <5	12,7%	12,2%	4,2%	0,9%	0,6%	0,0%	0,3%	0,1%
pos rate 5-14	18,0%	9,9%	4,2%	0,7%	0,7%	0,0%	0,5%	0,3%
pos rate >15	4,4%	2,5%	0,8%	0,1%	0,2%	0,0%	0,4%	0,2%

7.2.3 Health facility data

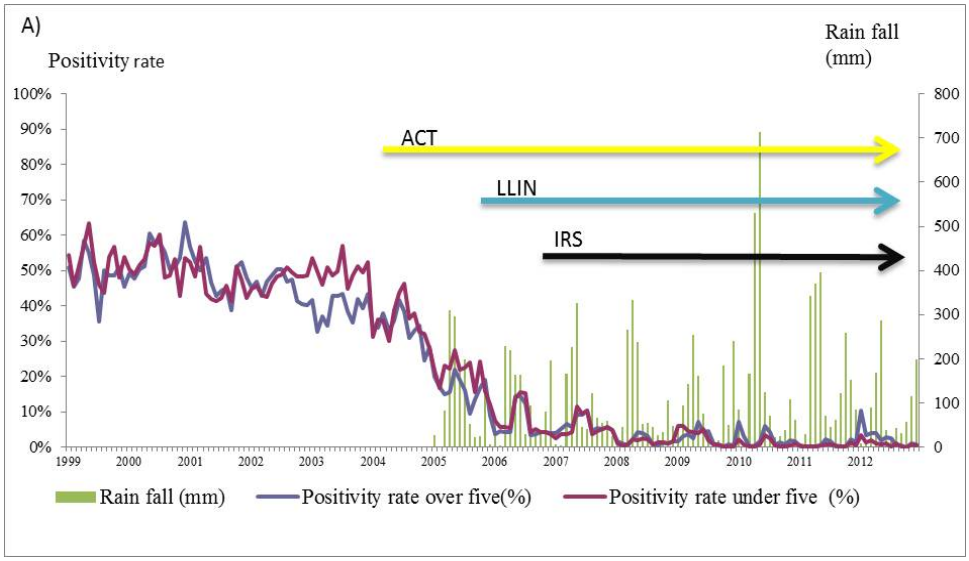
Following the introduction of RDT, the mean malaria-testing rates of all outpatients between 1999 and 2005 in Micheweni and North between 2006 and 2012 increased from 11% and 7% to 22% and 24%, respectively.

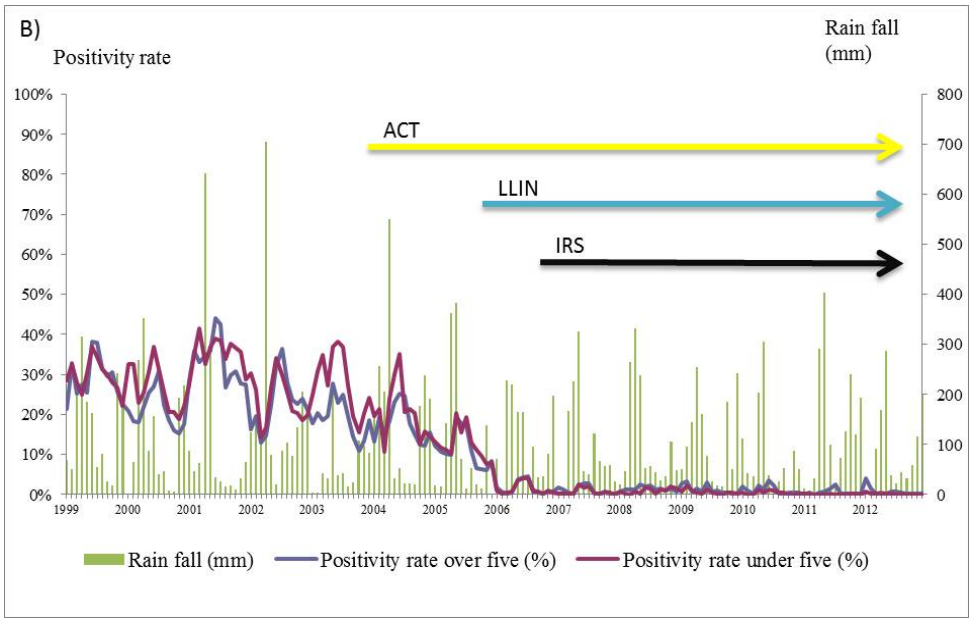
The parasitologically confirmed outpatient malaria diagnoses reported between January 1999 and December 2012 are presented by month in Figure 11 (a and b). The overall mean reduction in blood slide/RDT positivity rate between 2002 and 2012 was 96.0% in the two districts. Similarly to the trends in community based parasite prevalences, the main reduction occurred after September 2005 when vector control was implemented in addition to ACT. From 2008 onwards relatively steady blood slide/RDT positivity rates were observed with averages of 1.9% in Micheweni and 0.8% in North A.

7.2.4 Rainfall and malaria

No major changes in the annual rainfall were observed in North A between 1999 and 2012, except for 2003 and 2010 (Figure 11, a and b). The mean annual rainfalls for these two years were 42% and 20% lower than the mean annual rainfall (1219 mm) for the remaining years but this had no influence on the confirmed malaria positivity rates among fever patients. A significant correlation was however observed between monthly rainfalls and confirmed malaria diagnoses between 2007 and 2012 ([rp] =0.37, $p<0.01$), whereas no such correlation was observed during the pre-intervention and early malaria intervention periods (1999-2002 and 2003-2006) ([rp] =0.04, $p=0.78$ and [rp] = 0.33, $p=0.11$).

Figure 11: Positivity rates (confirmed malaria infection/total number of tested) among <5 and ≥5 febrile patients in public health care facilities in relation to monthly rainfall and introduction of ACT, LLIN and IRS in a) Micheweni and b) North A district between 1999 and 2012.

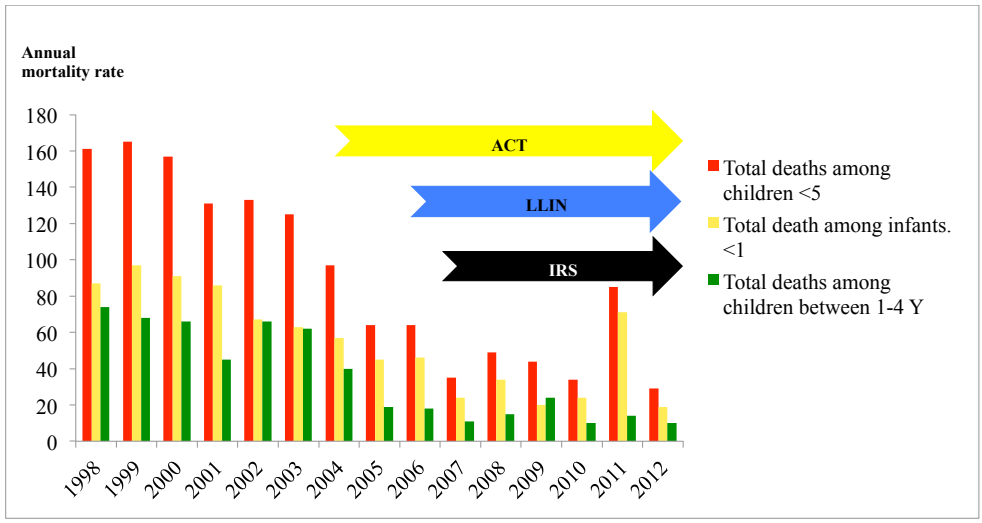




7.2.5 Crude child mortality

Figure 12 shows decline of all cause mortality figures among children under five by 70% in North A district between 1998 and 2012. The observed decrease was most pronounced in children aged 1-4 years with a reduction from an average of 64 to 14 reported deaths per year between 1998-2002 and 2007-2012, equivalent to a 78% reduction in absolute numbers. For children <1 year the corresponding reduction was 60% from 86 to 34 deaths.

Figure 12: Crude under-five, infant (<1y) and 1-4 y mortality between 1998 and 2012 in North A district



7.2.6 Entomological findings

Between 2005 and 2012, the strengthened vector control was associated with 99% reduction of the human biting rate (HBR). Generally, in recent years, there has been an increasingly marked seasonality with over 80% of mosquitoes collected during the three months of rainy season. We also observed a relative shift towards outdoor biting *An. arabiences* becoming the predominant malaria vector.

7.2.7 Vector control coverage

The overall ITN use among children under five in Zanzibar in 2005 was at 40%. Use of ITN among children under five in Micheweni district was only 10% which was the lowest rate in Zanzibar. Following the previously described LLIN mass distribution campaigns in Zanzibar, the bed net coverage among under- five children increased as shown in table 3. No statistically significant difference in LLIN usage between Micheweni district (218/322 (68%)) and North A district (241/338 (71%)) ($p = 0.45$) was found.

IRS coverage was higher than bed-net coverage; while 85% (503/660) of all children under five were reported to have slept under a treated bed net the night before the survey, effective IRS coverage of the children under five was 95% (638/675).

Equity analysis showed a similar effective coverage of LLIN in the poorest income group (70%) and the least poor (69%) but a tendency of a higher effective coverage of IRS in the least poor (99%) compared with the poorest (93%) was seen, however the difference was not statistically significant ($p = 0.055$)

Twenty-five per cent (125/508) of the caretakers reported seasonal use of bed-nets by their under-five children.

Table 3: Effective coverage of vector control intervention among children under five in Micheweni and North A district

Vector control intervention	Micheweni	North A	Total
Bed net (ITN or LLIN)	236/322 (73%)	267/338 (79%)	503/660 (85%)
IRS	314/333 (94%)	324/342 (95%)	638/675 (95%)
At least one of the interventions (IRS or bed net)	307/314 (98%)	321/339 (98%)	628/643(98%)

7.2.8 Caretakers' perceptions on malaria and vector control tools

Eighty-seven percent of the caretakers felt that the malaria burden had been declined compared to five years before and many of them (66%) did not see malaria as a serious health problem. Nevertheless, 83% of caretakers viewed children to be the age group most at risk of malaria infection.

Use of bed-nets and IRS was mentioned by 41% and 31% of the caretakers as the reason for the decline of malaria burden. Most caretakers (74%) viewed bed-nets as the best tool for preventing malaria. The best advantage of IRS was believed to be mosquito and general insect reduction. A minority (20%) of the caretakers mentioned side effects of IRS such as itching and increased mosquito and insect populations (such as bed bugs).

7.2.9 Sustainability

A vast majority of the caretakers (95%) said that they would continue to use malaria preventive measures for their under-five children. Ninety-three per cent recognized the significance of continuing bed net use and 89% of caretakers also stated the importance of continues use of IRS.

7.3 DISCUSSION

A major decline in malaria transmission in Zanzibar following intensified malaria control interventions was observed. This was most pronounced from 2004 to 2007 whereafter a persistent low-level transmission with seasonal increases emerged. The most significant impact on malaria prevalence and incidence was achieved after the intensified vector control with LLIN and IRS whereas the reduction of crude mortality was observed after the introduction of ACT.

7.3.1 Sustainability of high effective coverage of malaria control interventions

Overall good accesss to public health care as well as a continuous supply and adherence to RDTs and ACTs (**56, 201, 202**) have supported a continued efficient management of clinical malaria episodes in all age groups in Zanzibar.

Both studies (I & II) confirm a high and equitable effective coverage of and adherence to vector control measures (LLIN and IRS) in both districts following free mass distribution of bed net and IRS campaigns.

Maintained access to bed net for reaching high effective coverage is a challenge. Three mass-distribution campaigns in Zanzibar (2006, 2009 and 2012) have resulted in a sustained and relative high effective coverage of bed-nets. Both studies show that children under five use ITN/LLIN more than individual ≥5 years but the use of ITN/LLIN in all age groups in Micheweni and North A was still relatively high (around 70% between 2006 and 2013). This should be seen with regards to the assessment report on the feasibility of malaria elimination in Zanzibar which due to Zanzibar's high transmission potential and outbreak risk recommends an effective coverage of LLINs of at least 75% for reaching malaria elimination within a decade (**203**).

Our findings in study II show that the dramatic decline of malaria in Zanzibar was well understood by the caretakers. However, the caretakers' low malaria risk perception did not influence their use of LLIN negatively as was previously suggested in other studies in Vanuatu (**204-206**). The caretakers highly appreciated LLIN as a malaria prevention method. The coverage of IRS was higher than that of bed-nets, but IRS was a little less acknowledged for malaria prevention compared to LLIN. There were also some caretakers who mentioned few disadvantages of the IRS such as itching, and increase of insects which has also been reported from another study where DDT was used for IRS (**207**). Factors influencing community members to adhere to these interventions remain unclear. However, sustained high coverage of vector control tools is an important element of avoiding malaria resurgence and further reaching malaria elimination in Zanzibar. Access to these intervention measures is possible by maintaining their effective delivery by the government. Both studies show that

ZAMEP has achieved a sustained high effective coverage of malaria control interventions by a strong commitment of the Government of Zanzibar and external donors as well as high degree of community acceptance and involvement.

7.3.2 The impact of combined malaria control measures on malaria epidemiology

Following the deployment of the malaria control interventions in Zanzibar a significant reduction of malaria prevalence, confirmed malaria cases and crude under-five child mortality was observed. During the past recent years, significant reduction of malaria burden in several countries in sub-Saharan Africa has been reported (Table 4). However, the overall reductions in these areas were often less pronounced than in Zanzibar. There may be several reasons for this but we believe a main difference is the higher population-level uptake of the interventions in Zanzibar. Interestingly, there is a recent report of similar reduction of malaria transmission in a village in Senegal as in Zanzibar after apparent high access to preventive and curative interventions (**8**).

Using PCR for parasite detection in the cross sectional surveys, revealed a major reservoir of low-density asymptomatic parasitaemias across all age groups. Similar results have been seen in other low malaria transmission areas (**108, 208, 209**). An important impact of these low-density parasitaemias is that they may contribute significantly to the residual on-going transmission (**108, 210, 211**).

Our study provides data on a significant reduction of all cause child mortality coherent with the timing of the introduction of ACT. A similar decrease in crude child mortality was also observed on Bioko Island after massive malaria control interventions (**213**). However, in addition to the dramatic decline of malaria transmission in Zanzibar, other factors such as improved breastfeeding practices, IMCI, high immunization coverage and some improvement of the economical situation in Zanzibar cannot be fully ruled out (**186, 189, 212**). The strong impact on mortality may be explained by the reduced number of severe malaria episodes (following general improvement of malaria case management) but also by general reduction of malaria infections being a risk factor for severe manifestations of other accompanying bacterial infections, e.g. septicaemia (**214, 215**).

Table 4: Reported impact of different malaria control interventions in several different regions in Africa (2006-2014)

Country /setting	Observation period	Methods	Malaria control interventions	Outcome(s)
Five health facilities in different regions in The Gambia	1999-2007	Retrospective analysis of health facility records	Three fold increase in the proportion of children sleeping under ITN Malaria treatment with ACT (free of charge)	SPR declined by 73% MAD reduced by 100% MA reduced by 74%
Zambia	2003-2008	Program evaluation using different data sources such as national surveys, special studies and in-country reports	Sleeping under ITN increased by 75% No. of HH sprayed with IRS increased by 66% Use of ACT for malaria treatment	53% reduction of SPR and 68% reduction of severe anaemia in children under five. Mortality in infants and children aged 1-4 years decreased by 38% and 36%, respectively
Kilifi (Kenya)	1990-2007	Retrospective analysis of hospital data	Replacement of chloroquine with SP in 1998 and later with ACT in 2006 for malaria treatment (free of charge). The bed net coverage per person was doubled.	16-fold reduction of malaria prevalence. MA and MAD declined by 80%
Dielmo village (Senegal)	2007-2010	Longitudinal monitoring of inhabitants	Distribution of LLIN among inhabitants where no LLIN was used before. Use of ACT for malaria treatment	Malaria incidence decreased from 5.45 per 100 person-months to 0.41 but increased back to 4.57 due to development of vector resistance against pyrethroid.
30 district hospitals in Rwanda	2005-2010	Retrospective analysis of hospital and program data	Sleeping under ITN increased from near zero to 76% ACT deployment for malaria treatment	Over 50% decline in SPR, MA and MAD
Two villages in North east Tanzania	2003-2008	Annual cross sectional surveys	Training village health workers in malaria case management Proportion of people sleeping under ITN in both sides reached to >60%	Malaria prevalence reduction by ca. 85%
Several regions in Ethiopia and Rwanda	2001-2007	Retrospective analysis of health facility data	Large scale distribution of LLIN and use of ACT for malaria treatment	MA and MAD reduction in children under five by 55% and 67% in Rwanda and by 73% and 62% in Ethiopia, respectively

São Tomé e Príncipe	2005-2007	Retrospective analysis of health facility data and malariometric surveys conducted by the CNE	IRS Free distribution of LLIN Use of ACT for malaria treatment and SP for IPTp.	MA and MAD reduction by 80% and 95% respectively. Malaria prevalence reduction by 93%
The Bioko Island (Equatorial Guinea)	2003-2005	Household surveys and retrospective analysis of data from one health facility	IRS (86% of surveyed household were sprayed with IRS) IPTp	Malaria prevalence and confirmed malaria cases reduced by 32% and 14% respectively
Zanzibar (Study I)	1999-2013	Temporal trends of different malariometric indices in two districts with a population of approximately 200.000 people	Sleeping under LLIN increased by 22% and 61% in respective district to 70% 85% of all HH were sprayed with IRS Use of ACT free of charge & SP for IPTp	Malaria prevalence reduced by 97% Confirmed malaria cases reduced by 95% Reduction of the annual under-five mortality rate by 70%

(**4, 7, 9, 11-13, 158**)

(SPR= Slide positivity rate, MAD= Malaria attributed death, MA= Malaria admission, ACT= Artemisinin-based combination therapy, HH= Household, ITN= Insecticide treated bed net, LLIN= Long lasting insecticide treated nets, IRS= Indoor residual spraying, SP= Sulfadoxine-pyrimethamine, IPTp= Intermittent preventive treatment in pregnancy, RDT= Rapid diagnostic test, CNE= Centro National de Endemias)

Our findings highlights the following important changes in malaria transmission following implementing malaria control strategies in Zanzibar:

a) Malaria pre-elimination status was achieved already in 2007 after the rapid reduction of malaria transmission between 2004 and 2007 as described above.
b) Malaria transmission has changed towards more seasonal transmission. In addition, a geographically more heterogeneous transmission where malaria cases are distributed unevenly in few identifiable hotspots has occurred (**217**). These hotspots may be a main driving force of the present malaria transmission (**218**).
c) The annual parasitologically confirmed malaria positivity rate after 2008 has been fluctuating between 1% and 2% despite a sustained and relative high effective coverage of malaria control interventions.
d) After 2008, an increased proportion of confirmed malaria cases were seen among patients above 5 years of age. A similar shift in age among PCR detected low-density parasitaemias was not found. Malaria immunity is expected to decline as transmission reduces. This may result in a relative increase of infections in age groups that were previously protected through repeated exposure. Additionally, older age groups remain outside in the evenings and use LLIN less than children under five and therefore they are exposed more to outdoor biting mosquitoes. A similar shift in age

among clinical malaria episodes has been documented along with reduced transmission in Kenya (**7**).

Recently, development of pyrethroid resistance was observed on Pemba Island (**156**). Moreover, all vector control measures are directed towards indoor biting/resting mosquitoes. Following wide scale use of LLINs In Zanzibar, like several areas of sub-Saharan Africa (**151-154**) a shift in anopheline species towards outdoor biting/resting mosquitoes (*An. arabiensis*) has been observed. Therefore, despite the major reduction of HBRs a major reason for a halt in reduced transmission may be attributed to this change in mosquito population.

Finally, imported malaria from other areas in mainland has been mentioned as the main source for malaria transmission in Zanzibar based on modelling (**219**). In contrast to this report we believe that the bulk of the residual transmission appears still to take place within Zanzibar as travelling outside Zanzibar was reported by relatively few of malaria infected patients and can thus account for only a minor portion of the overall malaria incidence. Other malaria risk factors were not sleeping under LLINs or not having IRS recently performed. This suggests that transmission is still taking place indoors and promoted compliance to bed net use should still be required.

Zanzibar has reached the state of low malaria transmission but low-density parasitaemias, entomological changes, malaria transmission hot spots as well as difficulties in increasing high effective coverage of vector control measures are among challenges Zanzibar is facing for further decline of malaria transmission. Introduction of active case detection i.e. “an immediate investigation of detected malaria cases at health facilities through visits to patients’ homes and screening of approximately 100 neighbouring households to identify additional cases” (**203**) and IRS (carbamate) specifically targeting identified hotspots as well as use of single dose primaquine (for additional reduction of gametocytes) together with ACT are some of the new efforts introduced in Zanzibar addressing the above mentioned challenges. In addition to an increased level of effective coverage of vector control measures; use of sensitive molecular diagnostics for mass screen and targeted mass drug administration including seasonal malaria chemoprevention especially with regards to hot spots and risk groups are among other important actions needed for reaching malaria elimination (**220, 221**). As existing interventions will largely have to be maintained and new interventions must be added, an even greater need of financial support for avoiding malaria resurgence and to reach a malaria free Zanzibar is crucial.

7.4 LIMITATIONS

Study I

Unlike other malariometric data we could not triangulate morality data with data from other sources. In the past decade a general reduction of child mortality in Sub-Saharan Africa and Tanzania in particular has been observed. Nevertheless, the decline of child mortality in North A was coincided with the introduction of ACT. Furthermore, there are other studies that have shown the correlation between reduction of malaria burden and reduced child mortality.

The study was conducted in two districts of Zanzibar covering 20% of the whole population. Although there are differences in demography and socioeconomic condition between different parts of Zanzibar, the two district can be seen as representative of the isles with regards to the demography, climate, distribution of health services and so on.

Finally we did not have access to rainfall data between 1998 and 2005 in Micheweni; neither did we have vital statistic data from this district.

Study II

Use and wish to use malaria prevention measures may have been overestimated due to "desirability bias". This bias may have been increased due to the fact that the survey was identified with the ZMCP and the interviewers were health professionals. However, in order to minimize this risk, the importance of creating a comfortable environment during the interview was emphesized. Moreover, results from other studies indicate a similar and even higher rate of use of LLIN enhancing the validity of the findings in this study (**201 and study I**).

8 PART TWO

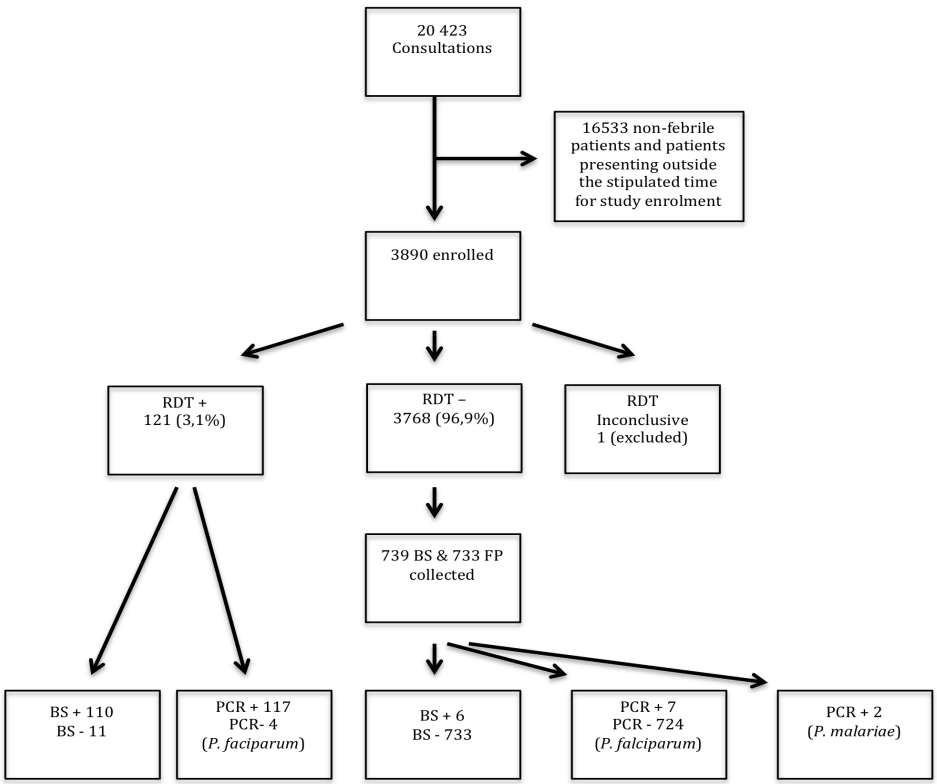
8.1 MATERIALS AND METHODS

8.1.1 Study population sampling and data collection

Study III

We enrolled 3890 patients from 12 primary health care facilities in Micheweni and North A districts between of May-July 2010. The process of patients' enrolment and study flow chart is shown in figure 13.

Figure 13: Study flow chart



(BS=blood smear, FP=filter paper, PCR=polymerase chain reaction)

Adherence to RDT results was the primary endpoint and the base for sample size calculation. Based on the previous study in Zanzibar, we assumed that approximately 10% of the RDT negative patients are prescribed ACT (**56**). Considering a potential clustering effect on health facility level we calculated that a sample of minimum 3054 patients would allow for an intra-cluster coefficient of slightly over 0.01 and still fulfil the given level of precision.

The participants in this study included patients aged ≥ 2 month, with fever (axillary temperature ≥ 37.5°C or history of fever during the preceding 24 hours) and who did not have symptoms of severe disease. Enrolled patients were tested with RDT. All RDT positive patients and randomly selected 20% of all RDT negative patients were additionally tested for malaria microscopy (thick BS). Further, approximately 100 µl blood on filter paper (Whatmann 3 MM) for additional molecular analysis were collected from these patients. All children under five were managed according to the IMCI guidelines. Malaria management was carried out according to the latest national treatment guidelines (**222**). Antibiotics and antipyretic medicines as well as other essential drugs (among children under five essential drugs were according to IMCI) were provided to all patients free of charge. Participation in this study included only one visit with no active follow up, however patients were encouraged by health workers to come back if there was no improvement of their condition or if their condition was deteriorated. Follow-up visit for children under five was recommended in accordance to the IMCI guidelines.

Upon enrolment in the study basic demographic data, previous anti-malarial treatment (in case of pregnancy use of IPTp), use of treated bed net (ITN/LLIN) the night before the survey as well as travel history was registered.

Study IV

The samples used in this study (RDTs and FPs) were all collected among febrile patients who were enrolled in study III.

8.1.2 Laboratory and molecular methodologies

Study III

8.1.2.1 RDT

Malaria diagnosis was done using the HRP-2 based Paracheck-pf® RDTs (Orchid Biomedical Systems, Goa India) which by the time of the study was the RDT device deployed by ZMCP. Performance and interpretation of the test was done in accordance to the manufacturer's instruction.

8.1.2.2 BS microscopy

Preparation of the BS was done on site as descried in part one. Discordancy in BS results between the two readers (positive versus negative, difference in species diagnosis or a difference of 50% in parasite density), were sent to Karolinska Institutet, Stockholm, Sweden for re-examination by a third expert microscopist. This was applied to all BS from patients with discrepancies between RDT and PCR results, RDT and BS results or PCR and BS results.

8.1.2.3 FP

Approximately 100 µL of blood was collected on filter papers (Whatmann 3 MM). These were dried thoroughly, put in individual zipped plastic bags containing desiccant and stored in room temperature (ca. 25° C) in Zanzibar. After completion of the study, all samples were

transported to Sweden for further molecular analysis.

8.1.2.4 DNA extraction

DNA was extracted from three (Ø 3 mm) filter paper punches (equivalent to ca. 10-15 µl whole blood) from all filter papers using a modified version of the ABI 6100 Nucleic Acid Prep Station protocol (Applied Biosystems, Fresno, CA) (**223**). When there were discrepancies between RDT and PCR results, we used Chelex-100 method for DNA re-extraction (**224**).

8.1.2.5 PCR

Samples belonging to all 121 RDT positive samples were analysed using *P. falciparum* specific nested PCR-Restriction Fragmental Length Polymorphism (RFLP) for detection of the following SNPs on specific drug resistance markers in *P. falciparum* parasites: N86Y, Y184F and D1246Y (*pfmdr1*) and: K76T (*pfcr*t) (**128, 225, 226**). Blood samples from all 733 RDT negative patients were pooled two by two where presence of *Plasmodium* DNA was screened with 18S ribosomal DNA (found in all five Plasmodium species) real-time PCR. A cut-off of 42 cycles was used to determine positive samples which were selected for species identification (**227**). Samples with discrepant RDT and PCR results were once again analysed using nested PCR analysis targeting *Plasmodium* cytochrome b (**224**).

Any PCR positive case which positivity was confirmed by another PCR method or by parasite detection by microscopy was defined as PCR positive. PCR analysis for HRP-2 deletion was made for all negative RDT samples with positive BS and/or PCR (**228**).

Study IV

8.1.2.6 RDT

For the purpose of DNA extraction from the RDTs, we compared DNA extraction from RDTs used in study III Paracheck-Pf® with Pf HRP-2/pan-LDH based RDT, SD-bioline Malaria Ag P.f/Pan (Standard diagnostic, Inc, USA) which is the RDT product used in Zanzibar since 2011that detects *P. falciparum* as well as non *falciparum* infections and mixed infections caused by both *P. falciparum* and other species.

8.1.2.7 Preparation of Plasmodium falciparum in vitro samples

In order to compare the detection limit of parasite DNA extracted from the two different RDT devices and FP samples, serial dilution of in-vitro cultured *P. falciparum* parasites was prepared. The parasite concentration in the culture was estimated by microscopical examination of giemsa stained thin films. Parasite cultures and malaria negative whole blood were lysed by freeze-thawing prior to serial dilution with parasite cultures and malaria negative whole blood.

8.1.2.8 DNA extraction

For the purpose of DNA extraction, the two RDT brands, were seeded with 5 µL blood of parasite concentration from 200,000 to 0,02/ µL which were derived from serial dilution as described above.

For comparison, FP was seeded in parallel with 5 µL (approximately equivalent to one 3-mm punch from the FP) of the serial dilutions. Before the extraction DNA cassettes were opened using a metal scapula and the nitrocellulose strip was into 3 mm pieces using scissors. DNA was then extracted from the distal two-third nitrocellulose strip of the RDTs containing approximately 5µL blood. Three different DNA extraction methods were evaluated

1) Simple elusion method (**102**)

2) Chelex-100 (**229**)

3) A modified version of extraction of ABI-6100 protocol (**223**)

DNA extraction from field samples were made from paired RDT and FP using the ABI PRISM 6100 Nucleic Acid PrepStation™ method.

8.1.2.9 PCR

All PCRs were run in parallel on DNA extracted from RDT and filter paper. PCR analyses for field samples were done using the PCR methods described in study III. Evaluation of the *P. falciparum* DNA extraction methods from RDTs and FPs was conducted by analysis of *P. falciparum* detection limits using three PCR techniques: 1) 18S ribosomal DNA (rDNA) nested PCR (**106**), 2) cytochrome b nested PCR (**107**) and 3)18S rDNA probe-based real-time PCR (**230**). The same volume of DNA was used from each extraction method (2-5 µL depending on the PCR method). The *P. falciparum* detection limits were determined as the lowest consecutive positive sample in the dilution series.

8.1.2.10 Data management and statistical analysis

Data from both study III and IV were double entered in CSPro and transferred to Microsoft Excel for validation. The statistical analyses were made in STATA 12 software. All frequencies, proportions and odds ratios were calculated with 95% CIs and corresponding *p*-values.

Study III

Adherence to RDT results was the primary endpoint and was defined as prescription and absence of prescription of anti-malarial drugs in RDT positive and negative patients, respectively.

Secondary endpoints included a) sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) of RDT compared with both PCR and BS microscopy and b) performance of RDT within IMCI in Zanzibar assessed as a sub-group analysis in children under five.

Since blood sampling for BS microscopy and PCR among RDT negative patients only included a random sample of 20%, we multiplied the absolute number of observations in these groups with a factors of 5.14 and 5.01 in all calculations of RDT sensitivity and specificity against PCR and microscopy, respectively. The corresponding CIs were, however, based on the true sample size. Statistical significances were stated at the 5% level.

Study IV

The outcomes of the SNP genotyping between extracted DNA from RDT and FP were compared by kappa analysis (κ). Since there were many incomplete RDT/PCR pairs, *pfmdr1* copy number variations were compared using Wilcoxon rank-sum test. Statistical significance was defined as $p < 0.05$.

8.2 RESULTS

Study III

Among 3890 enrolled patients, 121 (3.1%) were tested positive for malaria by HRP-2 based RDT. While 2212 (56.9%) of the total 3890 patients were female, 65 (53.7%) of all RDT positive patients were male. Children aged between 5-14 had the highest malaria positivity rate (6.1%, 32/528). Seventy-nine (65,3%) of all RDT positive patients were found in Micheweni district. However, the distribution of RDT positive patients was not even as 53 (67.1%) cases were detected at one health facility in Micheweni district.

Adherence to RDT test result was excellent. All 121 RDT positive, but only 3/3768 (0.1%) of RDT negative patients, (all aged >14 year) were prescribed anti-malarial medicine. Health workers' adherence to RDT test result was 99.9%.

Sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) of RDT compared with both PCR and BS microscopy is shown in table 5.

Overall RDT showed to have a relatively low sensitivity but very high specificity against both BS microscopy and PCR. Four out of six RDT negative but BS positive had high parasite densities (3573-50290 parasites/mL). We could not detect HRP-2 deletion in these samples. More importantly BS microscopy could not detect *P. falciparum* malaria infection among seven patients with both RDT and PCR positive result. Comparing BS and PCR resulted in nine BS negative but PCR positive cases.

Assessment of performance of malaria RDT within IMCI in Zanzibar showed no significant difference in children under five compared to other age groups (5-14 years and > 14 years). Even though the likelihood of RDT negative patients to receive antibiotics compared to RDT positive patients was statistically significant [OR 3.25 (95% CI 2.15–5.01)], among children under five, the rate of antibiotic prescription was similar between both groups i.e. 57% and 58% in the RDT negative and RDT positive group, respectively. Further, children under five were overall statistically significantly less likely to receive antipyretics than patients aged over 5 years [OR 0.22 (95% CI 0.19–0.26)].

Table 5: Sensitivity, specificity, PPV and NPV of RDT against PCR and BS microscopy

	PCR +	PCR –	Total	BS+	BS-	Total
RDT +	117	4	121	110	11	121
RDT -	7*	726*	733	6**	733**	739
Total	124	730	854	116	744	860
Sensitivity (95% CI)	76.5% (69.0- 83.9%)			78.6% (70.8–85.1)		
Specificity (95% CI)	99.9% (99.7- 100%)			99.7% (99.5-99.9%)		
Positive predictive value (95% CI)	96.7% (91.8- 99.1%)			91.7% (84.3-95.4%)		
Negative predictive value (95% CI)	99.0% (98.0- 99.6%)			99.2% (98.8-99.5%)		

*, **Multiplication with factors 5.14 and 5.01

Study IV

8.2.1 Sensitivity of RDT-DNA extraction methods in In vitro cultured parasites

The detection limit of *P. falciparum* DNA varied with RDT device and extraction method. Chelex-100 extraction performance was best for both RDT devices as well as for DNA extraction from FP with a detection limit of two parasites/μL. The detection limit of ABI extraction method was 10-fold higher (20 parasites/μL). Generally, the efficiency of DNA extraction from SD-Bioline Malaria Ag P.f/Pan was higher than from Paracheck-Pf ®. The simple elution method was unsuccessful for DNA extraction from Paracheck-Pf ®. DNA extraction from RDTs was generally equal to or better than DNA extraction from an equal volume (5 μL) of blood obtained from FP.

8.2.2 Parasite detection and drug resistance genotyping in field samples

No significant difference in PCR detection rates in DNA extracted from RDTs and FPs was found. Out of 855 paired RDT and FP field samples, 118 (13.8%; CI 95% 11.4-16.2%) were PCR positive in both groups of samples (κ=0.94). Among the RDT negative field samples (N=734), three (0.4%; CI 95% 0.0-0.9%) and six (0.8%; CI 95% 0.1-1.5%) were PCR positive from RDT and filter paper extracted DNA, respectively. Among the 121 RDT positive field samples, 115 (95.0%; CI 95% 91.1-99.0%) and 112 (92.6%; CI 95% 87.8-97.4%) were PCR positive (κ =0.50). No observed difference was found in the ability of detecting low-density parasitaemia (<100 parasites/μ L), statistical significance could not be calculated due to small number of low parasitaemias (only 12).

No significance differences in PCR success rates and genotyping in outcomes for the respective SNPs in all RDT positive samples were found as shown in table 4. Further, we could not detect multiple *Pfmdr1* copy number in any sample.

Table 6: PCR success rates and agreement of genotyping outcomes in field samples

	RDT PCR success rates **N = 121 (%; CI 95%)**	**Filter paper PCR success rates** **N = 121 (%; CI 95%)**	**Kappa value**
***Pfcrt* K76T**	114 (94.2; 89.9-98.5)	104 (86.0; 79.6-92.3)	0.72
***Pfmdr1* N86Y**	112 (92.6; 87.8-97.4)	109 (90.1; 84.6-95.5)	0.85
***Pfmdr1* Y184F**	110 (90.9; 85.7-96.2)	107 (88.4; 82.6-94.3)	0.74
***Pfmdr1* D1246Y**	113 (93.4; 88.8-97.9)	107 (88.4; 82.6-94.3)	0.77
***Pfmdr1* copy number**	84 (69.4; 61.0-77.8)	77 (63.6; 54.9-72.4)	-

8.3 DISCUSSION

Compared to the results in the previous study from Zanzibar when 30% of all fever cases were attributed to *P. falciparum* malaria (**56**), the sensitivity of RDT against BS microscopy has declined from 92% to 79% whereas the corresponded specificity has increased from 88% to 99%. The latter figure may reflect the new low malaria transmission context in Zanzibar but the observed low sensitivity of Paracheck-Pf ®, which was below the WHO-FIND (foundation of innovative new diagnostics) evaluation (**245**), is worrying since similar results have been reported from other studies (see Table 7). Also, considering the limitations of RDTs to detect low parasitaemias, it might not be the ultimate tool for malaria diagnosis in these settings (**231**).

The low sensitivity of Paracheck-Pf ® RDT can partly be explained by suboptimal performance of the test by the study health workers. All these patients had documented axillary temperature over 37.5 °C (37.6°C-39.8°C). Whilst two out of six RDT negative but BS and PCR positive cases had low parasite density (10 and 50 parasites/µl), the remaining four had high parasite densities of 3573, 14420, 25779 and 50190 parasites/mL, respectively. Four out of six false negative RDT results were reported from the same health facility that had the highest positivity rate among all health facilities in our study. This highlights the needs for improved systems for RDT supervision and quality control in primary health care facilities for RDT management. Other factors that can explain the low sensitivity of Paracheck-Pf ® RDT such as prozone effect i.e. false-negative test results due to antigen excess, could not be assessed retrospectively since blood was not available for serial dilution (**234**). Our study confirmed the previous observations from Zanzibar of an excellent adherence to RDT results among primary health workers (**56**) In contract to reports from various levels of health care and epidemiological settings in sub-Saharan Africa where health

workers' adherence to negative RDT result was very low (see table 7), our findings indicate that the health workers in this study had an excellent adherence to the test results despite the fact that 97 out of 100 tests were negative and resources for diagnosing alternative fever aetiologies were limited.

Furthermore, our study shows that RDT can be reliably integrated in the IMCI. There was neither any difference in RDT performance nor in adherence to RDT test results in children under five compared with other age groups. However, over 55% of antibiotic prescription rate among febrile children under five with negative RDT result was observed in this study. This is a concern, especially when data from similar settings show that the aetiology of these patients' febrile illnesses is due to viruses and not bacteria (**58 and Elfving et al, submitted**).

Table 7: Efficiency of and adherence to Rapid diagnostic test (RDT) results in different malaria endemic settings

Setting /country	Year	Type of RDT used	Dominant *Plasmodium* species	Sensitivity (versus Microscopy)	Specificity (versus Microscopy)	% of over-prescription of antimalarial drugs
Tanzania	2006-2007	Paracheck-Pf ®	*P. falciparum*	65%	88%	-
Tanzania	2007	ParaHIT *f* test (HRP-2 based)	*P. falciparum*	69% (Versus PCR)	100% (Versus PCR)	29%
Burkina Faso	2006	Paracheck-Pf ®	*P. falciparum*	NA	NA	80%
Malawi	2009	Four different products of HRP-2 based RDTs	*P. falciparum*	90%-97%	39%-68%	58%
Tanzania	2005	Paracheck-Pf ®	*P. falciparum*	95 %	96 %	54%
Zanzibar	2005	Paracheck-Pf ®	*P. falciparum*	92%	88%	0%
Zanzibar (Study III)	**2010**	Paracheck-Pf ®	***P. falciparum***	**79% vs. BS** **77% vs. PCR**	**100% vs. BS and PCR**	**0%**

(**56, 92, 93, 232, 233**)

(BS= Blood smear, PCR= Polymerase chain reaction)

In Study IV we assessed DNA extraction efficiency from two different malaria RDT devices and assessed the field applicability of RDT-DNA extraction for molecular surveillance, including detection of infections and key genetic markers associated with anti-malarial drug resistance. DNA extraction efficiency from in vitro cultured *P. falciparum* varied with RDT device and extraction method. Whilst simple elusion method showed to be unsuccessful for DNA extraction from Paracheck-Pf ®, using Chelex-100 and the modified version of extraction of ABI-6100 protocol were successful. Our results indicate that for low parasitaemias, Chelex-100 in combination with Cytochrome b nested PCR and 18S rDNA probe-based real-time PCR is the preferable methods of choice. The disadvantage of Chelex-100 method is that despite its relative labour intensity the quality of the DNA extracted is lower than ABI extraction method which is a high throughput method provides high quality DNA and a higher *P. falciparum* detection level compared to Chelex-100. Thus, ABI extraction could be suitable for analyses of RDT positive, symptomatic malaria patients with higher parasitaemia but this is an expensive method that requires special laboratory equipment. Finally, simple elution method is the cheapest and fastest alternative but its use may be limited by RDT design and choice of PCR.

The quality of the DNA extracted from RDTs collected in study III was equal to that extracted from FP, suggesting that RDT is a reliable alternative for DNA storage. In contract to FP, storage of RDT does not require drying, storage in plastic bags and use of desiccants. The same device used for malaria diagnosis in daily routine work can be used for both malaria case detection and as a reservoir of biological material without involving health workers and patients in difficulties associated with research trials. Hence, DNA extracted from RDTs collected from field can be used both for molecular surveillance as well as detection of low parasitaemias and for RDT quality control.

A disadvantage of RDT-DNA extraction is the limited amount of biological material (5– 15 μL blood). This makes RDT-DNA extraction a “one shot operation” with no possibilities for re-extraction. Using filter paper usually enables access to a much larger volume of blood (50–100 μL).

8.4 LIMITATIONS

Study III

In our study faint bands were interpreted as positive. A study from low malaria endemic area in Tanzania suggests that faint band should be regarded as negative especially in low malaria endemic setting (**235**) We did not make any examination of the health workers visual acuity, which may have influences the number of false negative results. Further although the adherence to the test result in the previous study (**56**) was good, the high adherence to RDT results in our study can be further explained by the extensive training all study health workers were provided prior to the start of the study. Another limitation of the study was that we only subjected 20% of all negative patients for further PCR and BS tests. Despite the statistical adjustments by using factor five in sensitivity and specificity calculation, it might be a weakness that we did not test all patients with BS and PCR.

Study IV

As the DNA extraction was conducted in accordance to the previously published methods, the same volume of elution buffer was not used in all the three extraction methods. The volume of elution buffer in the ABI method was four times higher compared to the simple elution method meaning that DNA was four times more diluted in the ABI method. A DNA quantification of the extracts with a qPCR method comparing the Ct-values may have had added more information to this study.

9 CONCLUSIONS

9.1 OVERALL CONCLUSION

During the conduct of the studies in this thesis, malaria elimination was not achieved in Zanzibar. However, following implementation of effective and sustainable tools and interventions with high coverage and uptake, Zanzibar has reached a state of malaria pre-elimination. Additional tools and interventions are necessary for further reduction of malaria transmission towards malaria elimination.

9.1.1 Specific conclusions

Study I

- Following implementation of combined malaria control interventions, Zanzibar has reached the state of malaria pre-elimination.

- Reducing malaria transmission further to achieve malaria elimination requires new tools and strategies including reorientation of the current malaria control activities with greater need of financial support.

Study II

- Effective coverage of vector control interventions in Zanzibar remains high despite the general perception of reduced malaria burden by the caretakers.

- Sustaining high effective coverage of vector control interventions, which is critical in reaching malaria elimination in Zanzibar, can be achieved by maintaining effective delivery of these interventions.

Study III

- The sensitivity of HRP-2 based RDT in the hands of primary health care workers compared with both PCR and microscopy for *P. falciparum* case detection was relatively low, highlighting the need for improved quality control of RDT use in primary health care facilities and more sensitive point-of-care malaria diagnostic tools in the new epidemiological context of low malaria transmission in Zanzibar.

- Adherence to test results with anti-malarial treatment was excellent. Further, the results show that RDT can be reliably integrated in IMCI as a tool for improved childhood fever management.

Study IV

- RDT is a valuable source of parasite DNA which can be used for improved malaria case detection, molecular drug resistance surveillance as well as RDT quality control.

- The purpose of DNA extraction should be considered when choosing which extraction method best suits the type of samples to be analysed.

10 PERSONAL REFLECTIONS

According to the WHO, the total sum of the international and domestic funding for malaria control and elimination in 2013 was only half of the total US$ 5.1 billion needed to achieve global targets for malaria control and elimination (**15**).

While we rightfully discuss the recent years' global successes in malaria control and feasibility of malaria elimination in some countries, we need to be reminded of the less favourable development in malaria control in some other countries in Africa.

The internal and regional conflicts have had devastating impact on the already depleted malaria situation in some regions in Africa. In ten countries in the Central Africa, the number of the malaria cases and admissions has increased compared to the previous years. (**15**). In The Central African Republic, the number of children who were sleeping under a mosquito net before the eruption of the recent internal conflict in 2013 was around 30% and access to malaria treatment was limited. Following the crisis the situation has deteriorated further as many malaria treatment centres were destroyed and health workers had to flee (**256**)

The situation in Zanzibar is much different from the above mentioned. Zanzibar's efforts in fighting malaria have been successful resulting in a rapid transition from a high to a low malaria endemic setting in less than a decade. Our studies show a good trust in the health services by the population with high adherence to vector control measures. The health workers too, show a high confidence in and adherence to the malaria treatment guidelines provided by ZAMEP. Further, the access to the public health facilities is relatively easy as the transport links in most areas in Zanzibar are good.

However, the feasibility of achieving malaria elimination must be assessed with regards to different characteristic conditions for different countries (**257**). In order to reach all malaria infections, Zanzibar needs to improve both the passive and active case detection by using more sensitive point-of-care diagnostic tools (**203**). Additionally, development of an effective system for detection and management of imported malaria cases as well as a sustained high coverage of the vector control measures are needed. All these interventions are laborious and costly. Having turned Zanzibar into an unstable low malaria endemic setting with non existing or low herd immunity, malaria resurgence would be devastating for Zanzibar and would rise questions whether a long-term malaria control is possible elsewhere in Africa (**158**, **191**). Therefore, adapting either the strategy of sustained control i.e. maintaining and marginally improving the current low malaria transmission status or setting up the goal of malaria elimination in a short term must be done cautiously and with regards to the feasibility of either strategies.

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12 REFERENCES

1. Frey C, Traore C, De Allegri M, Kouyate B, Muller O. Compliance of young children with ITN protection in rural Burkina Faso. Malaria journal. 2006;5:70.

2. Kwiatkowski, P. . How malaria has affected the human genome and what human genetics can teach us about malaria. Am J Hum Genet. 2005 Aug; 77(2):171-92.

3. Bhattarai A, Ali AS, Kachur SP, Martensson A, Abbas AK, Khatib R, et al. Impact of artemisinin-based combination therapy and insecticide-treated nets on malaria burden in Zanzibar. PLoS medicine. 2007;4(11):e309.

4. Serign J Ceesay, Climent Casals-Pascual, Jamie Erskine, Samuel E Anya, Nancy O Duah, Anthony J C Fulford, Sanie S S Sesay, Ismaela Abubakar,Samuel Dunyo, Omar Sey, Ayo Palmer, Malang Fofana, Tumani Corrah, Kalifa A Bojang, Hilton C Whittle, Brian M Greenwood, David J Conway. Changes in malaria indices between 1999 and 2007 in The Gambia: a retrospective analysis. Lancet 2008; 372: 1545–54

5. Ceesay SJ, Casals-Pascual C, Nwakanma DC, Walther M, Gomez-Escobar N, Fulford AJ, et al. Continued decline of malaria in The Gambia with implications for elimination. PloS one. 2010;5(8):e12242.

6. Chizema-Kawesha E, Miller JM, Steketee RW, Mukonka VM, Mukuka C, Mohamed AD, et al. Scaling up malaria control in Zambia: progress and impact 2005-2008. The American journal of tropical medicine and hygiene. 2010;83(3):480-8.

7. Wendy P O'Meara, Phillip Bejon, Tabitha W Mwangi, Emelda A Okiro, Norbert Peshu, Robert W Snow, Charles R J C Newton, Kevin Marsh. Eff ect of a fall in malaria transmission on morbidity and mortality in Kilifi , Kenya. Lancet 2008; 372: 1555–62

8. Trape J-F, Tall A, Sokhna C, Ly AB, Diagne N, Ndiath O, et al. The rise and fall of malaria in a west African rural community, Dielmo, Senegal, from 1990 to 2012: a 22 year longitudinal study. The Lancet Infectious Diseases. 2014;14(6):476-88.

9. Karema et al.: Trends in malaria cases, hospital admissions and deaths following scale-up of anti-malarial interventions, 2000–2010, Rwanda. Malaria Journal 2012 11:236

10. Noor AM, Kinyoki DK, Mundia CW, Kabaria CW, Mutua JW, Alegana VA, et al. The changing risk of Plasmodium falciparum malaria infection in Africa: 2000–10: a spatial and temporal analysis of transmission intensity. The Lancet. 2014;383(9930):1739-47.

11. Otten M, Aregawi M, Were W, Karema C, Medin A, Bekele W, et al. Initial evidence of reduction of malaria cases and deaths in Rwanda and Ethiopia due to rapid scale-up of malaria prevention and treatment. Malaria journal. 2009;8:14.

12. Hailay Desta Teklehaimanot , Awash Teklehaimanot , Anthony Kiszewski , Herodes Sacramento Rampao ,and Jeffrey D. Sachs. Malaria in São Tomé and Principe: On the Brink of Elimination after Three Years of Effective Antimalarial Measures. Am. J. Trop. Med. Hyg., 80(1), 2009, pp. 133–140

13. Immo Kleinschmidt, Brian Sharp, Luis E. Benavente, Chris Schwabe, Miguel Torrez, Jaime Kuklinski, Natashia Morris, Jaishree Raman, and Joseph Carter. Reduction in infection with Plasmodium falciparum one year after the introduction of malaria control interventions on Bioko Island, Equatorial Guinea. Am J Trop Med Hyg. 2006 Jun;74(6):972-8

14. Murray CJL, Rosenfeld LC, Lim SS, Andrews KG, Foreman KJ, Haring D, et al. Global malaria mortality between 1980 and 2010: a systematic analysis. The Lancet. 2012;379(9814):413-31.

15. WHO. World Malaria Report 2014. Available at: http://www.who.int/malaria/publications/world_malaria_report_2014/en/. (Accessed January 2015).

16. Sachs J, Malaney P. The economic and social burden of malaria. Nature. 2002 Feb 7;415(6872):680-5.

17. Masha F. Somi, James R. G. Butler, Farshid Vahid, Joseph Njau, S. Patrick Kachur, and Salim Abdulla.

Is There Evidence for Dual Causation Between Malaria and Socioeconomic Status?

Findings From Rural Tanzania. Am. J. Trop. Med. Hyg., 77(6), 2007, pp. 1020–1027

18. WHO. The global action plan for a malaria free world. 2008. Available at: http://www.rbm.who.int/gmap/ accessed January 2015.

19. Gwatkin, Davidson R. The burden of disease among the global poor: current situation, future trends, and implications for strategy. ISBN 0-8213-4619-9

20. Singh J, Desai MS, Pandav CS, Desai SP. Contributions of ancient Indian physicians-implications for modern times. J Postgrad Med. 2012 Jan-Mar;58(1):73-8.

21. Roncalli Amici R. The history of Italian parasitology. Vet Parasitol. 2001 Jul 12;98(1-3):3-30.

22. WHO. World malaria report, 2008. Available at: http://www.who.int/malaria/publications/atoz/9789241563697/en/, Accessed January 2015

23. Li J, Collins WE, Wirtz RA, Rathore D, Lal A, McCutchan TF. Geographic subdivision of the range of the malaria parasite Plasmodium vivax. Emerg Infect Dis. 2001 Jan-Feb;7(1):35-42.

24. Westling J, Yowell CA, Majer P, Erickson JW, Dame JB, Dunn BM. Plasmodium falciparum, P. vivax, and P. malariae: a comparison of the active site properties of plasmepsinscloned and expressed from three different species of the malaria parasite. Exp Parasitol. 1997 Nov;87(3):185-93.

25. White NJ. Plasmodium knowlesi: the fifth human malaria parasite. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2008;46(2):172-3.

26. Grigg MJ, William T, Drakeley CJ, Jelip J, von Seidlein L, Barber BE, et al. Factors that are associated with the risk of acquiring Plasmodium knowlesi malaria in Sabah, Malaysia: a case-control study protocol. BMJ open. 2014;4(8):e006004.

27. Paaijmans KP, Blanford S, Chan BH, Thomas MB. Warmer temperatures reduce the vectorial capacity of malaria mosquitoes. Biology letters. 2012;8(3):465-8.

28. Kaneko A. A community-directed strategy for sustainable malaria elimination on islands: short-term MDA integrated with ITNs and robust surveillance. Acta tropica. 2010;114(3):177-83.

29. Lyons et al.: Stable and fluctuating temperature effects on the development rate and survival of two malaria vectors, Anopheles arabiensis and Anopheles funestus. Parasites & Vectors 2013 6:104.

30. Cowman AF, Crabb BS. A parasite genome sheds light on an old enemy. Nat Biotechnol. 2002 Nov;20(11):1098-9.

31. Mendis K, Rietveld A, Warsame M, Bosman A, Greenwood B, Wernsdorfer WH. From malaria control to eradication: The WHO perspective. Tropical medicine & international health : TM & IH. 2009;14(7):802-9.

32. Kelly-Hope LA, McKenzie FE. The multiplicity of malaria transmission: a review of entomological inoculation rate measurements and methods across sub-Saharan Africa. Malaria journal. 2009;8:19.

33. WHO. Management of severe malaria, 2012. Available at: http://www.who.int/malaria/publications/atoz/9789241548526/en/ accessed in January 2015

34. Albrecht L, Angeletti D, Moll K, Blomqvist K, Valentini D, et al. (2014) B-Cell Epitopes in NTSDBL1a of PfEMP1 Recognized by Human Antibodies in Rosetting Plasmodium falciparum. PLoS ONE 9(12): e113248. doi:10.

1371/journal.pone.0113248

35. Adams Y, Kuhnrae P, Higgins MK, Ghumra A, Rowe JA. Rosetting Plasmodium falciparum-infected erythrocytes bind to human brain microvascular endothelial cells in vitro, demonstrating a dual adhesion phenotype mediated by distinct P. falciparum erythrocyte membrane protein 1 domains. Infection and immunity. 2014;82(3):949-59.

36. Carlson J, Helmby H, Hill AV, Brewster D, Greenwood BM, Wahlgren M. Human cerebral malaria: association with erythrocyte rosetting and lack of anti-rosetting antibodies. Lancet. 1990 Dec 15;336 (8729):1457-60.

37. Nacer A, Movila A, Sohet F, Girgis NM, Gundra UM, Loke P, et al. Experimental cerebral malaria pathogenesis-hemodynamics at the blood brain barrier. PLoS pathogens. 2014;10(12):e1004528.

38. MacPherson GG, Warrell MJ, White NJ, Looareesuwan S, Warrell DA. Human cerebral malaria. A quantitative ultrastructural analysis of parasitized erythrocyte sequestrastion. Am J Pathol. 1985 Jun;119(3):385-401.

39. van der Heyde HC, Nolan J, Combes V, Gramaglia I, Grau GE. A unified hypothesis for the genesis of cerebral malaria: sequestration, inflammation and hemostasis leading to microcirculatory dysfunction. Trends in parasitology. 2006;22(11):503-8.

40. Adams S, Brown H, Turner G. Breaking down the blood-brain barrier: signaling a path to cerebral malaria? Trends Parasitol. 2002 Aug;18(8):360-6.

41. Maitland K, Marsh K. Pathophysiology of severe malaria in children. Acta tropica. 2004;90(2):131-40.

42. Schumacher RF, Spinelli E. Malaria in children. Mediterranean journal of hematology and infectious diseases. 2012;4(1):e2012073.

43. Oluwayemi IO, Brown BJ, Oyedeji OA, Oluwayemi MA. Neurological sequelae in survivors of cerebral malaria. The Pan African medical journal. 2013;15:88.

44. Rogerson SJ, Mwapasa V, Meshnick SR. Malaria in pregnancy: linking immunity and pathogenesis to prevention. Am J Trop Med Hyg. 2007 Dec;77(6 Suppl):14-22.

45. Khunrae P, Higgins MK. Structural insights into chondroitin sulfate binding in pregnancy-associated malaria. Biochemical Society transactions. 2010;38(5):1337-41.

46. Nosten F, Rogerson SJ, Beeson JG, McGready R, Mutabingwa TK, Brabin B. Malaria in pregnancy and the endemicity spectrum: what can we learn? Trends in parasitology. 2004;20(9):425-32.

47. Mbonye AK, Bygbjerg IC, Magnussen P. Intermittent preventive of malaria in pregnancy: a new delivery system and its effect on maternal health and pregnancy outcomes in Uganda. Bulletin of the World Health Organization. 2008;86(2):93-100.

48. Michael English, Jeanine Puntl, Isaiah Mwangil, Kieran McHugh and Kevin Marsh. Clinical overlap between malaria and severe pneumonia in African children in hospital. Transactions of the royal society of tropical medicine and hygiene (1996) 90,658~662

49. Kallander K, Nsungwa-Sabiiti J, Balyeku A, Pariyo G, Tomson G, Peterson S. Home and community management of acute respiratory infections in children in eight Ugandan districts. Annals of tropical paediatrics. 2005;25(4):283-91.

50. Perkins BA, Zucker JR, Otieno J, Jafari HS, Paxton L, Redd SC, Nahlen BL, Schwartz B, Oloo AJ, Olango C, Gove S, Campbell CC. Evaluation of an algorithm for integrated management of childhood illness in an area of Kenya with high malariatransmission. Bull World Health Organ. 1997;75 Suppl 1:33-42.

51. Redd SC, Bloland PB, Kazembe PN, Patrick E, Tembenu R, Campbell CC Lancet. Usefulness of clinical case-definitions in guiding therapy for African children with malaria or pneumonia. 1992 Nov 7;340(8828):1140-3.

52. D'Acremont V, Lengeler C, Mshinda H, Mtasiwa D, Tanner M, Genton B. Time to move from presumptive malaria treatment to laboratory-confirmed diagnosis and treatment in African children with fever. PLoS medicine. 2009;6(1):e252.

53. English M, Reyburn H, Goodman C, Snow RW. Abandoning presumptive antimalarial treatment for febrile children aged less than five years--a case of running before we can walk? PLoS medicine. 2009;6(1):e1000015.

54. Bisoffi Z, Sirima BS, Angheben A, Lodesani C, Gobbi F, Tinto H, et al. Rapid malaria diagnostic tests vs. clinical management of malaria in rural Burkina Faso: safety and effect on clinical decisions. A randomized trial. Tropical medicine & international health : TM & IH. 2009;14(5):491-8.

55. d'Acremont V, Malila A, Swai N, Tillya R, Kahama-Maro J, Lengeler C, et al. Withholding antimalarials in febrile children who have a negative result for a rapid diagnostic test. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2010;51(5):506-11.

56. Msellem MI, Martensson A, Rotllant G, Bhattarai A, Stromberg J, Kahigwa E, et al. Influence of rapid malaria diagnostic tests on treatment and health outcome in fever patients, Zanzibar: a crossover validation study. PLoS medicine. 2009;6(4):e1000070.

57. Batwala V, Magnussen P, Nuwaha F. Antibiotic use among patients with febrile illness in a low malaria endemicity setting in Uganda. Malaria journal. 2011;10:377.

58. D'Acremont V, Kilowoko M, Kyungu E, Philipina S, Sangu W, Kahama-Maro J, et al. Beyond malaria--causes of fever in outpatient Tanzanian children. The New England journal of medicine. 2014;370(9):809-17.

59. Editorial. Integrated Management of Childhood Illnesses (IMCI) 1998 Journal of Tropical Pediatrics Vol.44 August 1998

60. S. Gove. Integrated management of childhood illness by outpatient health workers: technical basis and overviewBulletin of the World Health Organization, 1997, 75 (Supplement 1): 7-24

61. WHO, Handbook: IMCI integrated management of childhood illness, 2005. ISBN 92 4 154644 1

62. Bryce J, Victora CG, Habicht JP, Black RE, Scherpbier RW, Advisors M-IT. Programmatic pathways to child survival: results of a multi-country evaluation of Integrated Management of Childhood Illness. Health policy and planning. 2005;20 Suppl 1:i5-i17.

63. Adam T, Manzi F, Schellenberg JA, Mgalula L, de Savigny D, Evans DB. Does the Integrated Management of Childhood Illness cost more than routine care? Results from the United Republic of Tanzania. Bull World Health Organ. 2005 May;83(5):369-77.

65. Weber MW, Mulholland EK, Jaffar S, Troedsson H, Gove S, Greenwood BM. Bull World Health Organ. Evaluation of an algorithm for the integrated management of childhood illness in an area with seasonal malaria in the Gambia. 1997;75 Suppl 1:25-32.

66. WHO, Guidelines for the treatment of malaria. Second edition March 2010. Available at: http://www.who.int/malaria/publications/atoz/9789241547925/en/ accessed January 2015

67. WHO, Malaria microscopy quality assurance manual – Version 1. Available at: http://www.who.int/malaria/publications/atoz/mmicroscopy_qam/en/ accessed January 2015

68. CDC, TREATMENT GUIDELINES. Treatment of Malaria (Guidelines For Clinicians). Available at: http://www.cdc.gov/malaria/resources/pdf/clinicalguidance.pdf. Accessed January 2015

69. B. M. Greenwood and J. R. M. Armstrong . Comparison of two simple methods for determining malaria parasite density. Transactions of the Toyal Society of Tropical Medicine and Hygiene (1991) 85, 186-188

70. WHO, Basic malaria microscopy, 2010.available at: http://whqlibdoc.who.int/publications/2010/9789241547826_eng.pdf accessed january 2015

71. **WHO, New perspective malaria diagnosis. Available at http://www.who.int/tdr/publications/documents/malaria-diagnosis.pdf accessed January 2015**

72. **WHO, Malaria diagnosis: Memorandum from a WHO Meeting Bull World Health Organ. 1988; 66(5): 575–594.**

73. **David Bell and Rosanna W. Peeling. Evaluation of rapid diagnostic tests: malaria. *Nature Reviews Microbiology* , S34-S38 (September 2006)**

74. **The public health agency of Sweden, available at: http://www.folkhalsomyndigheten.se/amnesomraden/smittskydd-och-sjukdomar/smittsamma-sjukdomar/malaria/ accessed December 2014**

75. **WHO, Malaria rapid diagnosis - Making it work. Meeting report. 2003 available at: http://who.int/malaria/publications/atoz/rdt2/en/ accessed January 2015**

76. **Abeku TA, Kristan M, Jones C, Beard J, Mueller DH, Okia M, et al. Determinants of the accuracy of rapid diagnostic tests in malaria case management: evidence from low and moderate transmission settings in the East African highlands. Malaria journal. 2008;7:202.**

77. **Ojurongbe O, Adegbosin OO, Taiwo SS, Alli OA, Olowe OA, Ojurongbe TA, et al. Assessment of Clinical Diagnosis, Microscopy, Rapid Diagnostic Tests, and Polymerase Chain Reaction in the Diagnosis of Plasmodium falciparum in Nigeria. Malaria research and treatment. 2013;2013:308069.**

78. **Batwala V, Magnussen P, Nuwaha F. Are rapid diagnostic tests more accurate in diagnosis of plasmodium falciparum malaria compared to microscopy at rural health centres? Malaria journal. 2010;9:349.**

79. **Mayfong Mayxay', Sasithon Pukrittayakamee' , Kesinee Chotivanich', Sornchai Looareesuwan' and Nicholas J. White. Persistence of Plasmodium falciparum HRP-2 in successfully treated acute falciparum malaria. Transactions of the Toyal Society of Tropical Medicine and Hygiene (2001)9 5,179-182**

80. **Noedl H, Wernsdorfer WH, Miller RS, Wongsrichanalai C. Histidine-Rich Protein II: a Novel Approach to Malaria Drug Sensitivity Testing. Antimicrobial Agents and Chemotherapy. 2002;46(6):1658-64.**

81. **Mouatcho JC, Goldring JP. Malaria rapid diagnostic tests: challenges and prospects. Journal of medical microbiology. 2013;62(Pt 10):1491-505.**

82. **Fogg C, Twesigye R, Batwala V, Piola P, Nabasumba C, Kiguli J, et al. Assessment of three new parasite lactate dehydrogenase (pan-pLDH) tests for diagnosis of uncomplicated malaria. Transactions of the Royal Society of Tropical Medicine and Hygiene. 2008;102(1):25-31.**

83. **Makler MT, Piper RC, Milhous WK. Lactate dehydrogenase and the diagnosis of malaria. Parasitol Today. 1998 Sep;14(9):376-7.**

84. **Gerstl S, Dunkley S, Mukhtar A, De Smet M, Baker S, Maikere J. Assessment of two malaria rapid diagnostic tests in children under five years of age, with follow-up of false-positive pLDH test results, in a hyperendemic falciparum malaria area, Sierra Leone. Malaria journal. 2010;9:28.**

85. **Lee N, Baker J, Bell D, McCarthy J, Cheng Q. Assessing the genetic diversity of the aldolase genes of Plasmodium falciparum and Plasmodium vivax and its potential effect on performance of aldolase-detecting rapid diagnostic tests. Journal of clinical microbiology. 2006;44(12):4547-9.**

86. **Dzakah et al.: Plasmodium vivax aldolase-specific monoclonal antibodies and its application in clinical diagnosis of malaria infections in China. Malaria Journal 2013 12:199.**

87. **Moody A. Rapid Diagnostic Tests for Malaria Parasites. Clinical Microbiology Reviews. 2002;15(1):66-78.**

88. **Batwala V, Magnussen P, Hansen KS, Nuwaha F. Cost-effectiveness of malaria microscopy and rapid diagnostic tests versus presumptive diagnosis: implications for malaria control in Uganda. Malaria journal. 2011;10:372.**

89. **Shillcutt S, Morel C, Goodman C, Coleman P, Bell D, Whitty C, et al. Cost-effectiveness of malaria diagnostic methods in sub-Saharan Africa in an era of combination therapy. Bulletin of the World Health Organization. 2008;86(2):101-10.**

90. **Morris et al.: Rapid diagnostic tests for molecular surveillance of Plasmodium falciparum malaria -assessment of DNA extraction methods and field applicability. Malaria Journal 2013 12:106.**

91. **Iqbal J, Siddique A, Jameel M, Hira PR. Persistent histidine-rich protein 2, parasite lactate dehydrogenase, and panmalarial antigen reactivity after clearance of Plasmodium falciparum monoinfection. Journal of clinical microbiology. 2004;42(9):4237-41.**

92. **Chinkhumba J, Skarbinski J, Chilima B, Campbell C, Ewing V, San Joaquin M, et al. Comparative field performance and adherence to test results of four malaria rapid diagnostic tests among febrile patients more than five years of age in Blantyre, Malawi. Malaria journal. 2010;9:209.**

93. **Emanuele Nicastri , Nazario Bevilacqua, Monica Sañé Schepisi , Maria G. Paglia , Silvia Meschi , Shaali M. Ame , Jape A. Mohamed , Sabina Mangi , Robert Fumakule , Antonino Di Caro , Maria R. Capobianchi , Andrew Kitua, Fabrizio Molteni, Vincenzo Racalbuto, and Giuseppe Ippolito. Accuracy of Malaria Diagnosis by Microscopy, Rapid Diagnostic Test, and PCR Methods and Evidence of Antimalarial Overprescription in Non-Severe Febrile Patients in Two Tanzanian Hospitals**

Am. J. Trop. Med. Hyg., 80(5), 2009, pp. 712–717

94. **Thiam S, Thior M, Faye B, Ndiop M, Diouf ML, Diouf MB, et al. Major reduction in anti-malarial drug consumption in Senegal after nation-wide introduction of malaria rapid diagnostic tests. PloS one. 2011;6(4):e18419.**

95. **Bjorkman A, Martensson A. Risks and benefits of targeted malaria treatment based on rapid diagnostic test results. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2010;51(5):512-4.**

96. **Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM; Evidence of artemisinin-resistant malaria in western Cambodia. N Engl J Med. 2008 Dec 11;359(24):2619-20. doi: 10.1056/NEJMc0805011.**

97. **Noedl H, Socheat D, Satimai W. Artemisinin-resistant malaria in Asia. N Engl J Med. 2009 Jul 30;361(5):540-1. doi: 10.1056/NEJMc0900231.**

98. **Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin KM, Ariey F, Hanpithakpong W, Lee SJ, Ringwald P, Silamut K, Imwong M, Chotivanich K, Lim P, Herdman T, An SS, Yeung S, Singhasivanon P, Day NP, Lindegardh N, Socheat D, White NJ. Artemisinin resistance in Plasmodium falciparum malaria. N Engl J Med. 2009 Jul 30;361(5):455-67.**

99. **WHO, 2012. T3: Test. Treat. Track. Scaling up diagnostic testing, treatment and surveillance for malaria. Available at: http://www.who.int/malaria/publications/atoz/t3_brochure/en/accessed January 2015**

100. **Kim A Lindblade, Laura Steinhardt,Aaron Samuels, S Patrick Kachur and Laurence Slutsker. The silent threat: asymptomatic parasitaemia and malaria transmission**

Expert Rev. Anti Infect. Ther. 11(6), 623–639 (2013)

101. **Hwang et al.: Long-term storage limits PCR-based analyses of malaria parasites in archival dried blood spots. Malaria Journal 2012 11:339.**

102. **Cnops L, Boderie M, Gillet P, Van Esbroeck M, Jacobs J. Rapid diagnostic tests as a source of DNA for Plasmodium species-specific real-time PCR. Malaria journal. 2011;10:67.**

103. **Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N.**

Enzymatic amplification of beta-globulin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia. 1985. Biotechnology. 1992;24:476-80.

104. **Robert J. Henry and Agnelo Furtado (Editors). Cereal Genomics, Methods and Protocols. 2009. ISBN 978-1-62703-714-3**

105. **Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical chemistry. 2009;55(4):611-22.**

106. **Snounou G, Viriyakosol S, Jarra W, Thaithong S, Brown KN. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. Mol Biochem Parasitol. 1993 Apr;58(2):283-92.**

107. **Steenkeste N, Incardona S, Chy S, Duval L, Ekala MT, Lim P, et al. Towards high-throughput molecular detection of Plasmodium: new approaches and molecular markers. Malaria journal. 2009;8:86.**

108. **Okell LC, Bousema T, Griffin JT, Ouedraogo AL, Ghani AC, Drakeley CJ. Factors determining the occurrence of submicroscopic malaria infections and their relevance for control. Nature communications. 2012;3:1237.**

109. **Proux S, Suwanarusk R, Barends M, Zwang J, Price RN, Leimanis M, et al. Considerations on the use of nucleic acid-based amplification for malaria parasite detection. Malaria journal. 2011;10:323.**

110. **Bereczky S[1], Mårtensson A, Gil JP, Färnert A.**
Short report: Rapid DNA extraction from archive blood spots on filter paper for genotyping of

Plasmodium falciparum. Am J Trop Med Hyg. 2005 Mar;72(3):249-51.

111. **Mårtensson A, Strömberg J, Sisowath C, Msellem MI, Gil JP, Montgomery SM, Olliaro P, Ali AS, Björkman A. Efficacy of artesunate plus amodiaquine versus that of artemether-lumefantrine for the treatment of uncomplicated childhood Plasmodium falciparum malaria in Zanzibar, Tanzania. Clin Infect Dis. 2005 Oct 15;41(8):1079-86. Epub 2005 Sep 13.**

112. **Hopkins H, Gonzalez IJ, Polley SD, Angutoko P, Ategeka J, Asiimwe C, et al. Highly sensitive detection of malaria parasitaemia in a malaria-endemic setting: performance of a new loop-mediated isothermal amplification kit in a remote clinic in Uganda. The Journal of infectious diseases. 2013;208(4):645-52.**

113. **Aydin-Schmidt B, Xu W, Gonzalez IJ, Polley SD, Bell D, Shakely D, et al. Loop mediated isothermal amplification (LAMP) accurately detects malaria DNA from filter paper blood samples of low density parasitaemias. PloS one. 2014;9(8):e103905.**

114. **Corran P, Coleman P, Riley E, Drakeley C. Serology: a robust indicator of malaria transmission intensity? Trends in parasitology. 2007;23(12):575-82.**

115. **Hempelmann and Krafts: Bad air, amulets and mosquitoes: 2,000 years of changing perspectives on malaria. Malaria Journal 2013 12:232.**

116. **Krafts K, Hempelmann E, Skorska-Stania A. From methylene blue to chloroquine: a brief review of the development of an antimalarial therapy. Parasitology research. 2012;111(1):1-6.**

117. **Peters W. Drug resistance in malaria a perspective. Transactions of the Royal Society of**

Tropical Medicine and Hygiene. vol. 63. no. 1. 1969.

118. **T.T. Hien and N.J White. Qinghaosu. Lancet. 1993 Mar 6;341(8845):603-8**

119. **Cui L, Su XZ. Discovery, mechanisms of action and combination therapy of artemisinin. Expert Rev Anti Infect Ther. 2009 Oct;7(8):999-1013. doi: 10.1586/eri.09.68.**

120. **Tu Y. The discovery of artemisinin (qinghaosu) and gifts from Chinese medicine. Nature medicine. 2011;17(10):1217-20.**

121. **A. N. CHAWIRA, D. C. WARHURST, B. L. ROBINSON AND W. PETERS. The effect of combinations of qinghaosu (artemisinin) with standard antimalarial drugs in the suppressive treatment of malaria in mice. Transactions of the Royal Society of Tropical Medicine and Hygiene (1987) 81, 554-558**

122. R. N. Price, F. Nosten, C. Luxemburger M. van Vugt L. Phaipun', T. Chongsuphajaisiddhi

and N. J. White. Artesunate/mefloquine treatment of multi-drug resistant falciparum malaria. Transactions of the Royal Society of Tropical Medicine and Hygiene (1997) 91,574-577

123. White NJ, Nosten F, Looareesuwan S, Watkins WM, Marsh K, Snow RW, et al. Averting a malaria disaster. The Lancet. 1999;353(9168):1965-7.

124. N.J. White. Preventing antimalarial drug resistance through combinations. Drug Resist Updat. 1998 Mar;1(1):3-9.

125. Nicholas J. White, Michele van Vugtand Farkad Ezzet

Clinical Pharmacokinetics and Pharmacodynamics of Artemether-Lumefantrine. Clin Pharmacokinet 1999 Aug; 37 (2): 105-125

126. Bjorkman A, Bhattarai A. Public health impact of drug resistant Plasmodium falciparum malaria. Acta tropica. 2005;94(3):163-9.

127. Sisowath C, Strömberg J, Mårtensson A, Msellem M, Obondo C, Björkman A, Gil JP. In vivo selection of Plasmodium falciparum pfmdr1 86N coding alleles by artemether-lumefantrine (Coartem). J Infect Dis. 2005 Mar 15;191(6):1014-7. Epub 2005 Feb 8.

128. Holmgren G, Hamrin J, Svard J, Martensson A, Gil JP, Bjorkman A. Selection of pfmdr1 mutations after amodiaquine monotherapy and amodiaquine plus artemisinin combination therapy in East Africa. Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases. 2007;7(5):562-9.

129. Dahlstrom S, Ferreira PE, Veiga MI, Sedighi N, Wiklund L, Martensson A, et al. Plasmodium falciparum multidrug resistance protein 1 and artemisinin-based combination therapy in Africa. The Journal of infectious diseases. 2009;200(9):1456-64.

130. Martin RE, Marchetti RV, Cowan AI, Howitt SM, Bröer S, Kirk K. Chloroquine transport via the malaria parasite's chloroquine resistance transporter. Science. 2009 Sep 25;325(5948):1680-2. doi: 10.1126/science.1175667.

131. Valderramos SG, Valderramos JC, Musset L, Purcell LA, Mercereau-Puijalon O, Legrand E, et al. Identification of a mutant PfCRT-mediated chloroquine tolerance phenotype in Plasmodium falciparum. PLoS pathogens. 2010;6(5):e1000887.

132. R. N. Price, C. Cassar, Brockman, M. Duraisingh, M. Van Vugt,

N. J. White, F. Nosten, and S. Krishna. The pfmdr1 Gene Is Associated with a Multidrug-Resistant Phenotype in Plasmodium falciparum from the Western Border of Thailand. Antimicrob Agents Chemother. 1999 Dec;43(12):2943-9.

133. Mu J, Ferdig MT, Feng X, Joy DA, Duan J, Furuya T, et al. Multiple transporters associated with malaria parasite responses to chloroquine and quinine. Molecular Microbiology. 2003;49(4):977-89.

134. Noedl H, Se Y, Sriwichai S, Schaecher K, Teja-Isavadharm P, Smith B, et al. Artemisinin resistance in Cambodia: a clinical trial designed to address an emerging problem in Southeast Asia. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2010;51(11):e82-9.

135. Ashley EA, Dhorda M, Fairhurst RM, Amaratunga C, Lim P, Suon S, et al. Spread of artemisinin resistance in Plasmodium falciparum malaria. The New England journal of medicine. 2014;371(5):411-23.

136. Malmberg et al.: Temporal trends of molecular markers associated with artemether-lumefantrine tolerance/resistance in Bagamoyo district, Tanzania. Malaria Journal 2013 12:103.

137. Raghavendra K, Barik TK, Reddy BP, Sharma P, Dash AP. Malaria vector control: from past to future. Parasitology research. 2011;108(4):757-79.

138. WHO, 1982. Manual on environmental management for mosquito control with special emphasis on malaria vectors. Available at http://whqlibdoc.who.int/publications/1982/9241700661_eng.pdf accessed January 2016

139. Ghosh SK, Dash AP. Larvivorous fish against malaria vectors: a new outlook. Transactions of the Royal Society of Tropical Medicine and Hygiene. 2007;101(11):1063-4.

140. Rieckmann KH. The chequered history of malaria control: are new and better tools the ultimate answer? Annals of tropical medicine and parasitology. 2006;100(8):647-62.

141. Najera JA, Gonzalez-Silva M, Alonso PL. Some lessons for the future from the Global Malaria Eradication Programme (1955-1969). PLoS medicine. 2011;8(1):e1000412.

142. <WHO, 2003. Insecticide-treated

mosquito net interventions A manual for national control programme managers.Available at: file:///Users/delershakely/Downloads/mal-ITNinterventions_en%20(2).pdf accessed january 2015.

143. WHO, Guidelines for the use of the insectiside-treatmed mosquito nets for the prevention and control of malaria in Africa.1997.

144. Lengeler C. Insecticide-treated bed nets and curtains for preventing malaria. Cochrane Database Syst Rev. 2004

145. Lim SS, Fullman N, Stokes A, Ravishankar N, Masiye F, Murray CJ, et al. Net benefits: a multicountry analysis of observational data examining associations between insecticide-treated mosquito nets and health outcomes. PLoS medicine. 2011;8(9):e1001091.

146. Hawley WA, Phillips-Howard PA, ter Kuile FO, Terlouw DJ, Vulule JM, Ombok M, Nahlen BL, Gimnig JE, Kariuki SK, Kolczak MS, Hightower AW. Community-wide effects of permethrin-treated bed nets on child mortality and malaria morbidity in western Kenya. Am J Trop Med Hyg. 2003 Apr;68(4 Suppl):121-7.

147. Guillet P, Alnwick D, Cham MK, Neira M, Zaim M, Heymann D, Mukelabai K. Long-lasting treated mosquito nets: a breakthrough in malaria prevention. Bull World Health Organ. 2001;79(10):998.

148. Rehman et al.: Five years of malaria control in the continental region, Equatorial Guinea. Malaria Journal 2013 12:154.

149. Ngufor C, Tchicaya E, Koudou B, N'Fale S, Dabire R, Johnson P, et al. Combining organophosphate treated wall linings and long-lasting insecticidal nets for improved control of pyrethroid resistant Anopheles gambiae. PloS one. 2014;9(1):e83897.

150. Perry A. Investigations on the Mechanism of DDT Resistance in Certain Anopheline Mosquitos. Bull. Wld Hlth 1960, 22. 743-756 Org.

151. Mwangangi et al.: Shifts in malaria vector species composition and transmission dynamics along the Kenyan coast over the past 20 years. Malaria Journal 2013 12:13.

152. Derua et al.: Change in composition of the Anopheles gambiae complex and its possible implications for the transmission of malaria and lymphatic filariasis in north-eastern Tanzania. Malaria Journal 2012 11:188.

153. Bayoh MN, Mathias DK, Odiere MR, Mutuku FM, Kamau L, Gimnig JE, et al. Anopheles gambiae: historical population decline associated with regional distribution of insecticide-treated bed nets in western Nyanza Province, Kenya. Malaria journal. 2010;9:62.

154. Moiroux N, Gomez MB, Pennetier C, Elanga E, Djenontin A, Chandre F, et al. Changes in Anopheles funestus biting behavior following universal coverage of long-lasting insecticidal nets in Benin. The Journal of infectious diseases. 2012;206(10):1622-9.

155. Ndiath MO, Mazenot C, Sokhna C, Trape JF. How the malaria vector Anopheles gambiae adapts to the use of insecticide-treated nets by African populations. PloS one. 2014;9(6):e97700.

156. **Haji et al.: Challenges for malaria elimination in Zanzibar: pyrethroid resistance in malaria vectors and poor performance of long-lasting insecticide nets. Parasites & Vectors 20136:82.**

157. **Ranson H, N'Guessan R, Lines J, Moiroux N, Nkuni Z, Corbel V. Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? Trends in parasitology. 2011;27(2):91-8.**

158. **Trape J-F, Tall A, Diagne N, Ndiath O, Ly AB, Faye J, et al. Malaria morbidity and pyrethroid resistance after the introduction of insecticide-treated bednets and artemisinin-based combination therapies: a longitudinal study. The Lancet Infectious Diseases. 2011;11(12):925-32.**

159. **Smith JM. Mosquito nets: John Singer Sargent. JAMA. 2013 Jul 24;310(4):350-1. doi: 10.1001/jama.2013.5224.**

160. **Sabot O, Cohen JM, Hsiang MS, Kahn JG, Basu S, Tang L, et al. Costs and financial feasibility of malaria elimination. The Lancet. 2010;376(9752):1604-15.**

161. **Sinka ME, Bangs MJ, Manguin S, Coetzee M, Mbogo CM, Hemingway J, et al. The dominant Anopheles vectors of human malaria in Africa, Europe and the Middle East: occurrence data, distribution maps and bionomic precis. Parasites & vectors. 2010;3:117.**

162. **Carter R, Mendis KN. Evolutionary and Historical Aspects of the Burden of Malaria. Clinical Microbiology Reviews. 2002;15(4):564-94.**

163. **RBM, 2000. Framework for Monitoring Progress &Evaluating Outcomes and Impact. Avaialble at: http://whqlibdoc.who.int/hq/2000/WHO_CDS_RBM_2000.25.pdf. Accessed january 2015**

164. **WHO, 2008. Global malaria control and elimination: Report of a technical review. Available at: http://whqlibdoc.who.int/publications/2008/9789241596756_eng.pdf accessed January 2015**

165. **WHO, The Abuja Declaration and the Plan of Action, 2003.**

166. **UN, 2010. Millenium development goals at a glance. Available at: http://www.un.org/millenniumgoals/pdf/MDGs%20at%20a%20Glance%20SEPT%202010.pdf. Accessed January 2015**

167. **WHO, 2005. Fifty-eighth world health assembly available at: http://apps.who.int/gb/ebwha/pdf_files/WHA58-REC1/english/A58_2005_REC1-en.pdf. Accessed January 2015.**

168. **RBM, 2011. Refined/Updated GMAP Objectives, Targets, Milestones and Priorities Beyond 2011. Available at: http://www.rbm.who.int/gmap/gmap2011update.pdf. Accessed january 2015.**

169. **WHO, 2007. Malaria elimination: A field manual for low and moderate endemic countries. Available at: http://www.who.int/malaria/publications/atoz/9789241596084/en/accessed January 2015.**

170. **Tanner M, de Savigny D. Malaria eradication back on the table. Bulletin of the World Health Organization. 2008;86(2):82-.**

171. **Nájera JA, González-Silva M, Alonso PL (2011) Some Lessons for the Future from the Global Malaria Eradication Programme (1955–1969). PLoS Med 8(1): e1000412. doi:10.1371/journal.pmed.1000412**

172. **Cohen et al.: Malaria resurgence: a systematic review and assessment of its causes. Malaria Journal 2012 11:122.**

173. **WHO, 2014. From malaria control to malaria elimination: a manual for elimination scenario planning. ISBN 978 92 4 150702 8**

174. **Moonen B, Cohen JM, Snow RW, Slutsker L, Drakeley C, Smith DL, et al. Operational strategies to achieve and maintain malaria elimination. The Lancet. 2010;376(9752):1592-603.**

175. **Cotter C, Sturrock HJW, Hsiang MS, Liu J, Phillips AA, Hwang J, et al. The changing epidemiology of malaria elimination: new strategies for new challenges. The Lancet. 2013;382(9895):900-11.**

176. **Feachem RGA, Phillips AA, Targett GA, Snow RW. Call to action: priorities for malaria elimination. The Lancet. 2010;376(9752):1517-21.**

177. **Feachem RGA, Phillips AA, Hwang J, Cotter C, Wielgosz B, Greenwood BM, et al. Shrinking the malaria map: progress and prospects. The Lancet. 2010;376(9752):1566-78.**

178. **Beer N, Ali AS, de Savigny D, Al-Mafazy AW, Ramsan M, Abass AK, et al. System effectiveness of a targeted free mass distribution of long lasting insecticidal nets in Zanzibar, Tanzania. Malaria journal. 2010;9:173.**

179. **mal ERACGoHS, Operational R. A research agenda for malaria eradication: health systems and operational research. PLoS medicine. 2011;8(1):e1000397.**

180. **Lozano R, Soliz P, Gakidou E, Abbott-Klafter J, Feehan DM, Vidal C, et al. Benchmarking of performance of Mexican states with effective coverage. The Lancet. 2006;368(9548):1729-41.**

181. **Salim Abdulla, Joanna Armstrong Schellenberg, Rose Nathan, Oscar Mukasa, Tanya Marchant,**

Tom Smith, Marcel Tanner, Christian Lengeler. Impact on malaria morbidity of a programme supplying insecticide treated nets in children aged under 2 years in Tanzania: community cross sectional study BMJ 2001;322:270–3

182. **Barat LM, Palmer N, Basu S, Worrall E, Hanson K, Mills A.**
Do malaria control interventions reach the poor? A view through the equity lens. Am J Trop Med Hyg. 2004 Aug;71(2 Suppl):174-8.

183. **National Bureau of Statistics Ministry of Finance. The United Republic of Tanzania. Tanzania_in_figures2012. June, 2013.**

184. **National Bureau of Statistics Ministry of Finance, Dar es Salaam and Office of Chief Government Statistician. President's Office, Finance, Economy and Development Planning, Zanzibar 2012 Population and housing census, 2013.**

185. **Revolutionarry government of Znazibar, ministry of health and social welfare, Zanzibar health sector reform ZMoHSW 2006-2010. 2011**

186. **Office of Chief Government Statistician, Zanzibar Socio-Economic Survey 2012, 2013**

187. **Ministry of health, Zanzibar. MOH Health bulletin 2012. 2013**

188. **National Bureau of Statistics Ministry of Planning, Economy and Empowerment Dar es Salaam. Tanzania Census 2002, August, 2006**

189. **National Bureau of Statistics Dar es Salaam, Tanzania. Tanzania Demographic and Health Survey 2010. 2011**

190. **Eli Schwartz, Hedva Pener, Sultan M. Issa; and Jacob Golenser. An overview of the malaria situation in Zanzibar. Journal of Community Health, Vol. 22, No. 1, February 1997**

191. **Smith DL, Cohen JM, Moonen B, Tatem AJ, Sabot OJ, Ali A, Mugheiry SM. Infectious disease. Solving the Sisyphean problem of malaria in Zanzibar. Science. 2011 Jun 17;332(6036):1384-5. doi: 10.1126/science.1201398.**

192. **Ministry of health, Zanzibar. MOH Health bulletin 2007. 2008**

193. **Ministry of health, Zanzibar. MOH Health bulletin 2008. 2009**

194. **UCSF Global health sciences, The global health group, 2014. Background paper, the surveillance system to facilitate malaria elimaintion.**

195. Statistics Canada. 2010, ISBN 978-1-100-16410-6,

196. Sisowath C, Ferreira PE, Bustamante LY, Dahlstrom S, Martensson A, Bjorkman A, et al. The role of pfmdr1 in Plasmodium falciparum tolerance to artemether-lumefantrine in Africa. Tropical medicine & international health : TM & IH. 2007;12(6):736-42.

197. Stewart L, Gosling R, Griffin J, Gesase S, Campo J, Hashim R, et al. Rapid assessment of malaria transmission using age-specific sero-conversion rates. PloS one. 2009;4(6):e6083.

198. Drakeley CJ, Corran PH, Coleman PG, Tongren JE, McDonald SL, Carneiro I, et al. Estimating medium- and long-term trends in malaria transmission by using serological markers of malaria exposure. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(14):5108-13.

199. Cook J, Kleinschmidt I, Schwabe C, Nseng G, Bousema T, Corran PH, et al. Serological markers suggest heterogeneity of effectiveness of malaria control interventions on Bioko Island, equatorial Guinea. PloS one. 2011;6(9):e25137.

200. Filmer D, Pritchett LH. Estimating wealth effects without expenditure data--or tears: an application to educational enrollments in statesof India. Demography. 2001 Feb;38(1):115-32.

201. Shakely D, Elfving K, Aydin-Schmidt B, Msellem MI, Morris U, Omar R, et al. The usefulness of rapid diagnostic tests in the new context of low malaria transmission in Zanzibar. PloS one. 2013;8(9):e72912.

202. Fröberg et al.: Decreased prevalence of Plasmodium falciparum resistance markers to amodiaquine despite its wide scale use as ACT partner drug in Zanzibar. Malaria Journal 2012 11:321.

203. ZMCP, Malaria elimination in Zanzibar: A Feasibility Assessment. 2009

204. Atkinson JA, Fitzgerald L, Toaliu H, Taleo G, Tynan A, Whittaker M, et al. Community participation for malaria elimination in Tafea Province, Vanuatu: Part I. Maintaining motivation for prevention practices in the context of disappearing disease. Malaria journal. 2010;9:93.

205. Beer N, Ali AS, Eskilsson H, Jansson A, Abdul-Kadir FM, Rotllant-Estelrich G, et al. A qualitative study on caretakers' perceived need of bed-nets after reduced malaria transmission in Zanzibar, Tanzania. BMC public health. 2012;12:606.

206. Toe LP, Skovmand O, Dabire KR, Diabate A, Diallo Y, Guiguemde TR, et al. Decreased motivation in the use of insecticide-treated nets in a malaria endemic area in Burkina Faso. Malaria journal. 2009;8:175.

207. Rodríguez AD, Penilla RP, Rodríguez MH, Hemingway J, Trejo A, Hernández-Avila JE. Salud Publica Mex. Acceptability and perceived side effects of insecticide indoor residual spraying under different resistance management strategies. 2006 Jul-Aug;48(4):317-24

208. Harris I, Sharrock WW, Bain LM, Gray KA, Bobogare A, Boaz L, et al. A large proportion of asymptomatic Plasmodium infections with low and sub-microscopic parasite densities in the low transmission setting of Temotu Province, Solomon Islands: challenges for malaria diagnostics in an elimination setting. Malaria journal. 2010;9:254.

209. Manjurano A, Okell L, Lukindo T, Reyburn H, Olomi R, Roper C, et al. Association of sub-microscopic malaria parasite carriage with transmission intensity in north-eastern Tanzania. Malaria journal. 2011;10:370.

210. Ouedraogo AL, Bousema T, Schneider P, de Vlas SJ, Ilboudo-Sanogo E, Cuzin-Ouattara N, et al. Substantial contribution of submicroscopical Plasmodium falciparum gametocyte carriage to the infectious reservoir in an area of seasonal transmission. PloS one. 2009;4(12):e8410.

211. Schneider P, Bousema JT, Gouagna LC, Otieno S, van de Vegte-Bolmer M, Omar SA, Sauerwein RW.
Submicroscopic Plasmodium falciparum gametocyte densities frequently result in mosquito infection.Am J Trop Med Hyg. 2007 Mar;76(3):470-4.

212. The united republic of Tanzania, ministries of health, Zanzibar & mainland. MDGs GOAL 4, 5 AND 6. 2011

213. Kleinschmidt I, Schwabe C, Benavente L, et al. Marked increase in child survival after four years of intensive malaria control. *The American journal of tropical medicine and hygiene* 2009; 80(6): 882-8.

214. Molineaux L. Nature's experiment: what implications for malaria prevention? The Lancet. 1997;349(9066):1636-7.

215. Scott JAG, Berkley JA, Mwangi I, Ochola L, Uyoga S, Macharia A, et al. Relation between falciparum malaria and bacteraemia in Kenyan children: a population-based, case-control study and a longitudinal study. The Lancet. 2011;378(9799):1316-23.

216. Mmbando BP, Vestergaard LS, Kitua AY, Lemnge MM, Theander TG, Lusingu JP. A progressive declining in the burden of malaria in north-eastern Tanzania. Malaria journal. 2010;9:216.

217. Cook J, Xu W, Msellem M, Vonk M, Bergström B, Gosling R, Al-Mafazy A, McElroy P, Molteni F, Abass AK, Garimo I, Ramsan M, Ali A, Mårtensson A, Björkman A. Mass Screening and Treatment on the Basis of Results of a Plasmodium falciparum-Specific Rapid DiagnosticTest Did Not Reduce Malaria Incidence in Zanzibar. J Infect Dis. 2014 Nov 26. pii: jiu655. [Epub ahead of print]

218. Ahmed S, Galagan S, Scobie H, Khyang J, Prue CS, Khan WA, et al. Malaria hotspots drive hypoendemic transmission in the Chittagong Hill Districts of Bangladesh. PloS one. 2013;8(8):e69713.

219. Le Menach A, Tatem AJ, Cohen JM, Hay SI, Randell H, Patil AP, et al. Travel risk, malaria importation and malaria transmission in Zanzibar. Scientific reports. 2011;1:93.

220. Greenwood B. Review: Intermittent preventive treatment--a new approach to the prevention of malaria in children in areas with seasonal malaria transmission. Tropical medicine & international health : TM & IH. 2006;11(7):983-91.

221. Bousema T, Griffin JT, Sauerwein RW, Smith DL, Churcher TS, Takken W, et al. Hitting hotspots: spatial targeting of malaria for control and elimination. PLoS medicine. 2012;9(1):e1001165.

222. ZMCP, Standard treatment guidelines, 2010.

223. Dahlstrom S, Veiga MI, Ferreira P, Martensson A, Kaneko A, Andersson B, et al. Diversity of the sarco/endoplasmic reticulum Ca(2+)-ATPase orthologue of Plasmodium falciparum (PfATP6). Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases. 2008;8(3):340-5.

224. Hsiang MS, Lin M, Dokomajilar C, Kemere J, Pilcher CD, Dorsey G, et al. PCR-based pooling of dried blood spots for detection of malaria parasites: optimization and application to a cohort of Ugandan children. Journal of clinical microbiology. 2010;48(10):3539-43.

225. A molecular marker for chloroquine-resistant falciparum malaria. N Engl J Med. 2001 Jan 25;344(4):257-63.Djimdé A, Doumbo OK, Cortese JF, Kayentao K, Doumbo S, Diourté Y, Coulibaly D, Dicko A, Su XZ, Nomura T, Fidock DA, Wellems TE, Plowe CV.

226. Veiga MI, Ferreira PE, Bjorkman A, Gil JP. Multiplex PCR-RFLP methods for pfcrt, pfmdr1 and pfdhfr mutations in Plasmodium falciparum. Molecular and cellular probes. 2006;20(2):100-4.

227. Shokoples SE, Ndao M, Kowalewska-Grochowska K, Yanow SK. Multiplexed real-time PCR assay for discrimination of Plasmodium species with improved sensitivity for mixed infections. Journal of clinical microbiology. 2009;47(4):975-80.

228. Baker J, McCarthy J, Gatton M, Kyle DE, Belizario V, Luchavez J, Bell D, Cheng Q. Genetic diversity of Plasmodium falciparum histidine-rich protein 2 (PfHRP2) and its effect on the performance ofPfHRP2-based rapid diagnostic tests. J Infect Dis. 2005 Sep 1;192(5):870-7. Epub 2005 Jul 21.

229. **Ishengoma DS, Lwitiho S, Madebe RA, Nyagonde N, Persson O, Vestergaard LS, et al. Using rapid diagnostic tests as source of malaria parasite DNA for molecular analyses in the era of declining malaria prevalence. Malaria journal. 2011;10:6.**

230. **Kamau E, Tolbert LS, Kortepeter L, Pratt M, Nyakoe N, Muringo L, et al. Development of a highly sensitive genus-specific quantitative reverse transcriptase real-time PCR assay for detection and quantitation of plasmodium by amplifying RNA and DNA of the 18S rRNA genes. Journal of clinical microbiology. 2011;49(8):2946-53.**

231. **McMorrow ML, Aidoo M, Kachur SP. alaria rapid diagnostic tests in elimination settings--can they find the last parasite? Clin Microbiol Infect. 2011 Nov;17(11):1624-31. doi: 10.1111/j.1469-0691.2011.03639.x. Epub 2011 Sep 13.**

232. **McMorrow ML, Masanja MI, Abdulla SM, Kahigwa E, Kachur SP.Am J Trop Med Hyg. 2008 Sep;79(3):385-90.**
Challenges in routine implementation and quality control of rapid diagnostic tests for malaria--Rufiji District,Tanzania.

233. **Reyburn H, Mbakilwa H, Mwangi R, Mwerinde O, Olomi R, Drakeley C, et al. Rapid diagnostic tests compared with malaria microscopy for guiding outpatient treatment of febrile illness in Tanzania: randomised trial. Bmj. 2007;334(7590):403.**

234. **Maltha J, Gillet P, Jacobs J. Malaria rapid diagnostic tests in endemic settings. Clin Microbiol Infect. 2013 May;19(5):399-407. doi: 10.1111/1469-0691.12151. Epub 2013 Feb 25.**

235. **Allen LK, Hatfield JM, DeVetten G, Ho JC, Manyama M. Reducing malaria misdiagnosis: the importance of correctly interpreting Paracheck Pf(R) "faint test bands" in a low transmission area of Tanzania. BMC infectious diseases. 2011;11:308.**

236. **De Carvalho GB[1], de Carvalho GB. Duffy Blood Group System and the malaria adaptation process in humans. Rev Bras Hematol Hemoter. 2011;33(1):55-64. doi: 10.5581/1516-8484.20110016.**

237. **Warrell, D.A. and H.M- Gilles (2002) Essential Malariology. Fourth Edition.**

ISBN: 0340740647

238. **Metselaar D, Van Thiel PM (1959) Classification of malaria. Tropical and Geographic Medicine 11: 157–161.**

239. **John c. Beier, Gerry f. killeen, and John i. Githure. Short report: entomologic inoculation rates and Plasmodium falciparum malaria prevalence in Africa. Am. J. Trop. Med. Hyg., 61(1), 1999, pp. 109–113.**

240. **Henry J. Shikani, Brandi D. Freeman, Michael P. Lisanti, Louis M. Weiss, Herbert B. Tanowitz, and Mahalia S. Desruisseaux. Cerebral Malaria We Have Come a Long Way. The American Journal of Pathology, Vol. 181, No. 5, November 2012**

241. **Lalit Kumar Das, Bishwanath Padhi,and Sudhansu Sekar Sahu. Prediction of outcome of severe *falciparum* malaria in Koraput, Odisha, India: A hospital-based study. Trop Parasitol. 2014 Jul-Dec; 4(2): 105–110. doi: 10.4103/2229-5070.138538**

242. **WHO. Malaria in children under five available at: http://www.who.int/malaria/areas/high_risk_groups/children/en/ accessed Decmber 2014.**

243. **Luxemburger C, Ricci F, Nosten F, Raimond D, Bathet S, White NJ. The epidemiology of severe malaria in an area of low transmission in Thailand.Trans R Soc Trop Med Hyg. 1997 May-Jun;91(3):256-62.**

244. **WHO. 2013, Malaria in pregnant women, 2013, available at: http://www.who.int/malaria/areas/high_risk_groups/pregnancy/en/ accessed December 2014.**

245. **WHO-FIND Foundation of Innovative New Diagnostic, Malaria RDT Product Testing Reports Found 1-5 results of WHO product testing of malaria RDTs 2008-2013**

246. DNA isolation methods, available at http://science.marshall.edu/murraye/links%20for%20students/samantha%20qiagin%20method.pdf) Accessed December 2014.

247. Bing-Yuan Chen and Harry W. Janes (Editors) PCR cloning protocols (2002), Second edition.

248. Leo L.M. Poon, Bonnie W.Y. Wong, Edmund H.T. Ma, Kwok H. Chan, Larry M.C. Chow, Wimal Abeyewickreme, Noppadon Tangpukdee, Kwok Y. Yuen,Yi Guan, Sornchai Looareesuwan, and J.S. Malik Peiris. Sensitive and Inexpensive Molecular Test for Falciparum Malaria: Detecting Plasmodium falciparum DNA Directly from Heat-Treated Blood by Loop-Mediated Isothermal Amplification. Clinical Chemistry 52, No. 2, 2006

249. Kobayashi et al, 1999. Three Reforms" in China: Progress and Outlook Sakura. Japan research institute. Available at: http://www.jri.co.jp/english/periodical/rim/1999/RIMe199904threereforms/ accessed Decem,ber 2014.

250. Maureen Coetzee, 1987. A supplement to the anophelinae of Africa south of the Sahara (Afrotropical region) Isbn 062003213.

251. Map of Zanzibar, Wikipedia. Available at: http://upload.wikimedia.org/wikipedia/commons/thumb/8/84/Map_of_Zanzibar_Archipelago-en.svg/474px-Map_of_Zanzibar_Archipelago-en.svg.png accessed December 2014.

252. WHO. 2014, The partnership for Maternal, New born and Child health, available at: http://www.who.int/pmnch/activities/countries/tanzania/en/index1.html accessed November 2014

253. ZMCP. National guidelines for malaria diagnosis and treatment in Zanzibar, 2002.

254. WMA Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects, available at http://www.wma.net/en/30publications/10policies/b3/ accessed November, 2014;

255. Good clinical practice, ICH available at http://www.ich.org/products/guidelines.html accessed November, 2014).

256. UNICEF. 2014. Delivering mosquito nets to protect displaced children from malaria in the Central African Republic, 2014 available at: http://www.unicef.org/infobycountry/car_72777.html, accessed November 2014.

257. Andrew J Tatem, David L Smith, Peter W Gething, Caroline W Kabaria, Robert W Snow, Simon I Hay. Ranking of elimination feasibility between malaria-endemic Countries. Lancet 2010; 376: 1579–91

258. Stahl HD, Kemp DJ, Crewther PE, Scanlon DB, Woodrow G, Brown GV, Bianco AE, Anders RF, Coppel RL. Sequence of a cDNA encoding a small polymorphic histidine- and alanine-rich protein from Plasmodiumfalciparum. Nucleic Acids Res. 1985 Nov 11;13(21):7837-46.

259. WHO, 2001. Antimalarial drug combination therapy. Report of a WHO Technical Consultation. Available at: http://whqlibdoc.who.int/hq/2001/WHO_CDS_RBM_2001.35.pdf accessed January 2015.